

In Figure 4 are presented data on the effect of pentobarbital sodium on pH, CO_2 , and sodium of the serum of a dog that received a smaller dose of methyl salicylate. This animal showed only mild intoxication, with a respiratory rate of 66 per minute, and there were moderate alterations in the electrolyte structure of the blood. Ten minutes after the injection of pentobarbital sodium, the CO_2 tension had risen to 37 mm. Hg with corresponding changes of pH and CO_2 content. Forty-five minutes later, the values approached normal even closer. During the following hours of observation the respiratory depressant effect of pentobarbital sodium proved to be transient, and the respirations again increased in magnitude. Concomitantly, the CO_2 tension fell again, and the pH rose somewhat. Twenty-four hours later, the animal appeared to have recovered from all ill effects and presented normal values for all constituents of the plasma (not shown). The dog appeared to be narcotized, or perhaps comatose, for 12 hours after the injection of the pentobarbital sodium. This is noteworthy since the usual duration of the narcosis produced by such doses of pentobarbital sodium rarely exceeds 4 hours.

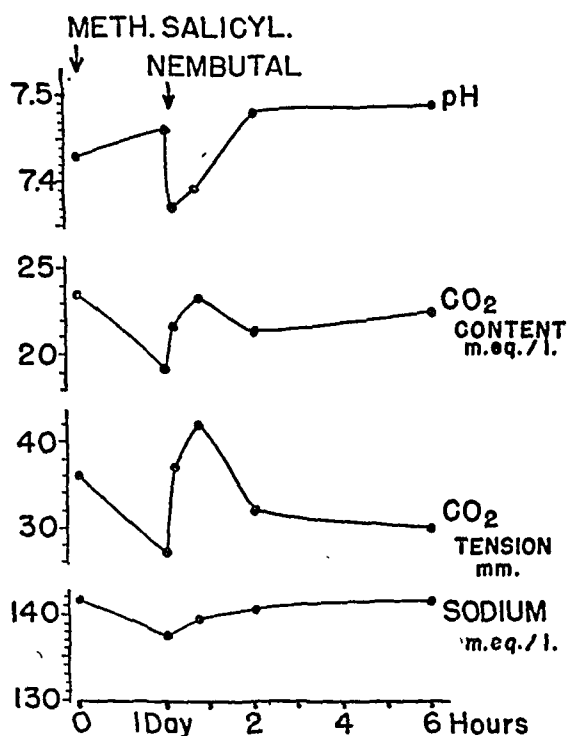


FIG. 4. THE EFFECT OF PENTOBARBITAL SODIUM

Dog 645, weighing 18 kgm., received 7 ml. of methyl salicylate on the day preceding the injection of 29 mgm. per kgm. of pentobarbital sodium (nembutal).

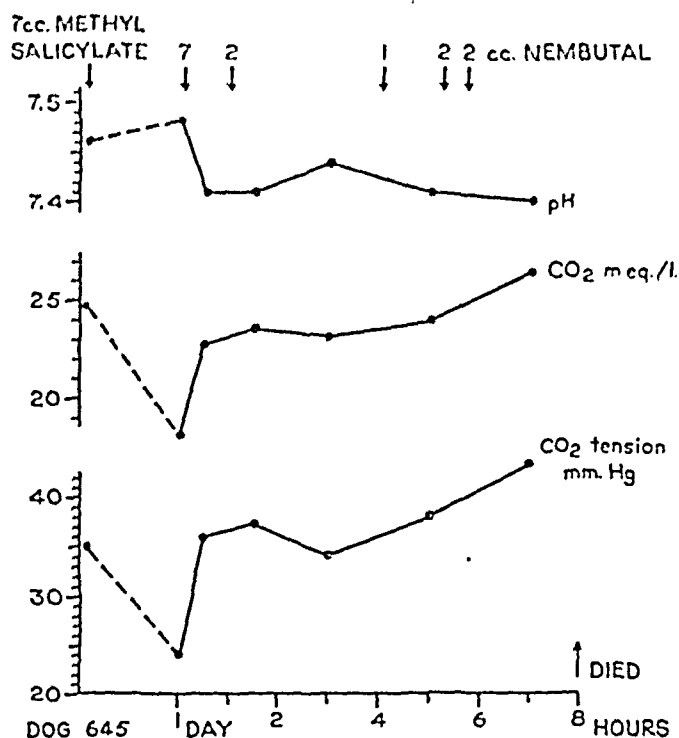


FIG. 5. THE EFFECT OF REPEATED INJECTIONS OF PENTOBARBITAL SODIUM

Dog 645, weighing 19 kgm., received 7 ml. of methyl salicylate on the day preceding the injections of pentobarbital sodium at intervals indicated.

In Figure 5 are shown data of an experiment designed to keep the respirations and the CO_2 tension of a dog with salicylism as nearly normal as possible by repeated injections of pentobarbital sodium. Dog 645 received 7 ml. of methyl salicylate, an amount usually producing only moderate intoxication. On the next day, a blood sample showed moderately reduced tension and content of CO_2 . At that time the respirations were fairly deep at a rate of about 40 per minute, and the hyperpnea appeared to be increasing. After the injection of the hypnotic drug, the respirations immediately slowed to 23 per minute. The blood sample drawn 30 minutes afterwards showed a return to normal values for pH and CO_2 . A short time later the respirations increased again, and 2 ml. of pentobarbital sodium were given, resulting in a diminution of the hyperpnea. Samples 1½ and 3 hours after the first injection indicated that a normal CO_2 tension was being maintained. After the fourth hour, the respirations of the dog increased again in frequency, but became much shallower. More pentobarbital sodium was given without any noticeable effect on the respirations which remained at a rate of about 60 per minute.

Bloods drawn at that time showed slowly-rising CO_2 tension. The animal became increasingly comatose and died after 7½ hours.

In Figure 6 are presented 2 experiments on the antagonistic effects of pentobarbital sodium and of sodium salicylate on dogs 839 and 841. The respiratory rate of both animals fell immediately after the injection of the hypnotic drug and the blood samples drawn 5 minutes later showed slight increases in the CO_2 tension. Then, the animals received 0.3 gram per kgm. of body weight of sodium salicylate slowly by vein. Half an hour later, the respiratory rate of dog 839 had in-

creased greatly, and a blood sample after 1 hour showed elevation of the pH with corresponding reduction of the CO_2 tension. Repeated injections of pentobarbital sodium did not reduce the respiratory rate, but the sample drawn 3 hours after the injection of salicylate showed a less marked deviation from the normal than the 1-hour sample. The animal was growing weaker and more comatose at that time and died 15 minutes after the last sample of blood was drawn. Dog 841 showed the suppressive effects of the hypnotic drug on the hyperpnea more clearly, the respirations remaining at a low frequency for more than ½ hour following the injection of the sodium salicylate. This animal recovered from the effects of both drugs.

Sodium barbital. One experiment was performed to test the effect of sodium barbital on the hyperpnea of salicylism. One dog received 0.1 gram per kgm. of the drug intraperitoneally on the day after the administration of 10 ml. of methyl salicylate. The respirations slowed progressively during the next 2 hours to a level of 40 per minute when the animal died.

The experiments with pentobarbital sodium offer support for the assumption of a primary effect of salicylates on the respiratory center. If salicylism were characterized by a metabolic acidosis, the hyperventilation would constitute a compensatory mechanism tending to lessen the fall in pH by reduction of the carbonic acid of the blood. Suppression of an hyperpnea of such origin would lead rapidly to low pH values and elevated tensions of CO_2 . Actually, in the foregoing experiments the pH and CO_2 tension returned immediately to normal values with abolition of the hyperpnea and did not drift into the acidotic range. The corrective effect of single injections of pentobarbital sodium on the ionic equilibrium was transient, but normal values of pH and CO_2 could be maintained by repeated doses. It appears that salicylate and pentobarbital sodium, while exhibiting antagonistic effects on the ventilation of the dogs, had synergistic depressant and toxic actions on their central nervous systems. The animals lapsed into a deeply comatose state and died after the combined administration of both drugs in doses that were easily tolerated singly.

Paraldehyde. In order to explore further the effects of hypnotic drugs on salicylism, 2 experi-

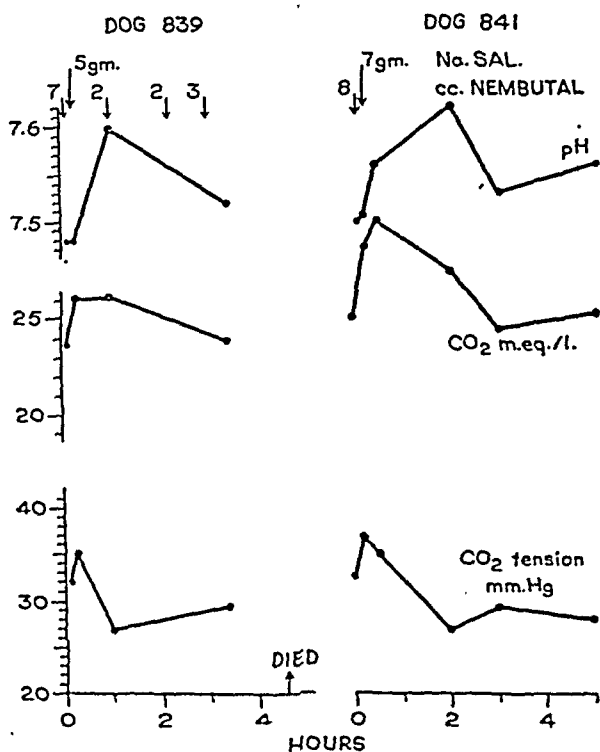


FIG. 6. THE EFFECTS OF SODIUM SALICYLATE AND OF PENTOBARBITAL SODIUM

Dog 839, weighing 16 kgm., received 7 ml. of the hypnotic drug. The respiratory rate fell from 24 to 14 per minute. The animal then received 5 grams of sodium salicylate in 25 per cent solution slowly by vein. The respiratory rate increased to 64 per minute and remained elevated throughout the experiment. Dog 841, weighing 19.5 kgm., received 8 ml. of pentobarbital sodium by vein. His respirations promptly decreased from 20 to 8 per minute. Seven ml. of sodium salicylate were then injected by vein. The respiratory rate remained constant for ½ hour, then increased to values of 72 per minute after 2 hours. After 7 hours, the rate had fallen again to 28 per minute. The animal remained comatose 9 hours

TABLE IV
Salicylism in dogs: effect of paraldehyde and of morphine

Dog number	Drug	pH	CO ₂	pCO ₂	Respiratory rate	Comment
		<i>m. eq. per liter</i>	<i>m. eq. per liter</i>	<i>mm. Hg</i>	<i>per minute</i>	
940	Paraldehyde	7.46	24.9	35	20	Preliminary sample, followed by intraperitoneal injection of 10 ml. methyl salicylate. Weight 18 kgm.
		7.46	16.1	22	100	After 23 hours; respiration moderately increased. 10 ml. paraldehyde injected after sample.
		7.48	17.0	22	70	½ hour later; deep respirations. 10 ml. paraldehyde injected after sample.
		7.47	11.0	14	160	2 hours later; died after 2 more hours.
941	Paraldehyde	7.40	22.1	36	20	Preliminary sample, followed by intraperitoneal injection of 10 ml. methyl salicylate.
		7.48	18.1	25	130	After 23 hours; panting intermittently. 10 ml. paraldehyde injected after sample.
		7.40	17.6	27	60	10 minutes later.
		7.52	12.8	15	250	1 hour later; panting, comatose.
		7.54	10.0	11	250	4 hours later; comatose, labored breathing; died ½ hour after sample.
Sp.	Morphine	7.46	14.5	19	200	16 hours after 10 ml. methyl salicylate. 135 mgm. morphine injected intramuscularly, in divided doses, over period of 2½ hours.
		7.57	12.0	13	180	3 hours after start of morphine injections; twitching of extremities every 3 to 4 minutes, generalized convulsions immediately after sample; died 2 hours later.

ments were carried out with paraldehyde. This drug was chosen because of its low toxicity and because it has little, if any, effect on the respiration. In Table IV are presented data on 2 dogs, each of which was injected with paraldehyde on the day following the administration of 10 ml. of methyl salicylate. Dog 940 received 20 ml. and dog 941 received 10 ml. of paraldehyde intraperitoneally. The data listed in the table demonstrate the absence of any depressant effect of paraldehyde on either hyperpnea or alkalosis. Actually, the impression was gained that the hyperpnea was intensified following the administration of the hypnotic drug. Both animals died about 4 hours after receiving doses of paraldehyde far below those tolerated by normal dogs. It would appear, therefore, that paraldehyde, as well as the barbiturates, potentiate the toxic effect of salicylates.

Morphine. In view of the pronounced depressant influence of morphine on the respiration of normal animals, it appeared of interest to study its effect on the hyperpnea of salicylism. The data of 1 experiment are presented in Table IV. Morphine did not produce significant reduction in either depth or rate of the respirations. Its outstanding pharmacologic effect was a further in-

crease of the hyper-reflexia, leading to generalized tetanic convulsions. In 2 other dogs, similar observations were obtained.

It is known that morphine has both narcotic and convulsant effects. This experiment suggests that salicylates have the property of reinforcing the convulsant and suppressing the hypnotic action of morphine.

The experiments with the several hypnotic and respiratory depressant drugs may indicate that salicylates increase the susceptibility of the central nervous system to the toxic effects of hypnotic drugs. The results suggest that the administration of any hypnotic drug in the treatment of salicylism in man is inadvisable.

EFFECT OF SODIUM BICARBONATE IN SALICYLISM

Since sodium bicarbonate has been thought to ameliorate the toxic effects of salicylates (8, 12, 18), it appeared of interest to study its effect on the ionic structure of the blood in salicylism. In Figure 7 are presented the data on 1 dog who received 0.6 gram per kgm. of sodium bicarbonate intravenously 1 day after the administration of 10 ml. of methyl salicylate. Two preliminary samples of blood drawn 4 hours apart indicated that the animal was in a state of moderate salicylism. Ten

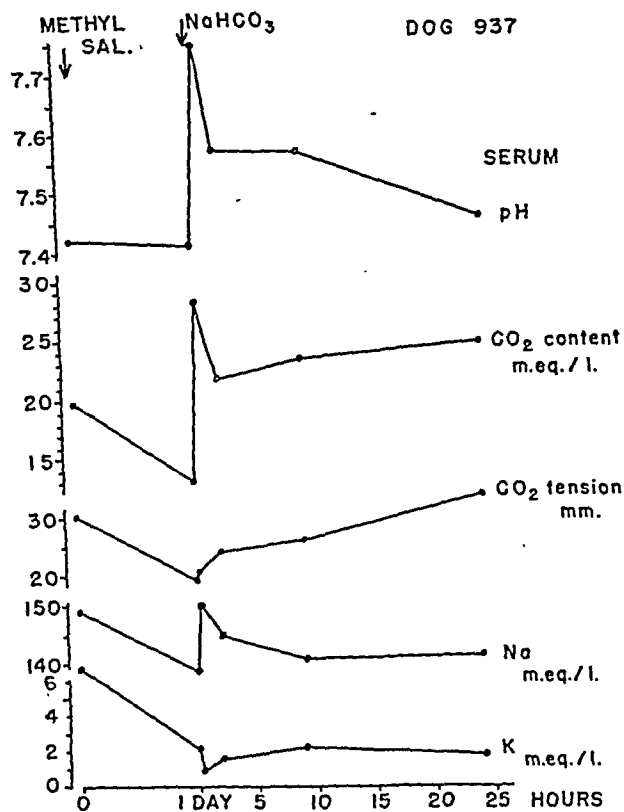


FIG. 7. THE EFFECT OF SODIUM BICARBONATE ON SALICYLISM

Dog 937, weighing 17 kgm., received 10 ml. of methyl salicylate intramuscularly on the day preceding the intravenous injection of 10 grams of sodium bicarbonate in 5 per cent solution. The animal appeared unaffected by the injection.

minutes after the injection of the sodium bicarbonate, the plasma pH was found to be 7.73, and the CO₂ content had increased by 15 m.eq. per liter, while the CO₂ tension had remained unchanged. The serum sodium, which was below the normal level before the injection, rose, while the potassium decreased even further. Blood samples 2 and 9 hours later showed that the pH had decreased and the CO₂ tension had risen slowly. Twenty-four hours after the injection of the sodium bicarbonate, the animal appeared to be entirely normal and showed no sign of intoxication. In Figure 8 are portrayed the changes in dog 938, which received identical experimental treatment. Five minutes after the injection of sodium bicarbonate, the blood pH rose to 7.68 and the CO₂ content to 31 m.eq. per liter. For 9 hours after the injection, a state of severe alkalosis persisted, but 24 hours

later the animal appeared to be in good condition. Of the several more experiments with similar results, one deserves special mention. Shortly after the administration of sodium bicarbonate the animal developed convulsions, apparently tetanic in nature, and died.

Sodium bicarbonate does not appear to have a primary effect on the disturbance of the electrolyte equilibrium in salicylism. Neither the hyperpnea nor the CO₂ tension were immediately changed. The bicarbonate content was increased. The pH changed in proportion to the varying ratio between bicarbonate and carbonic acid, a result similar to that obtained by two investigators (19) in

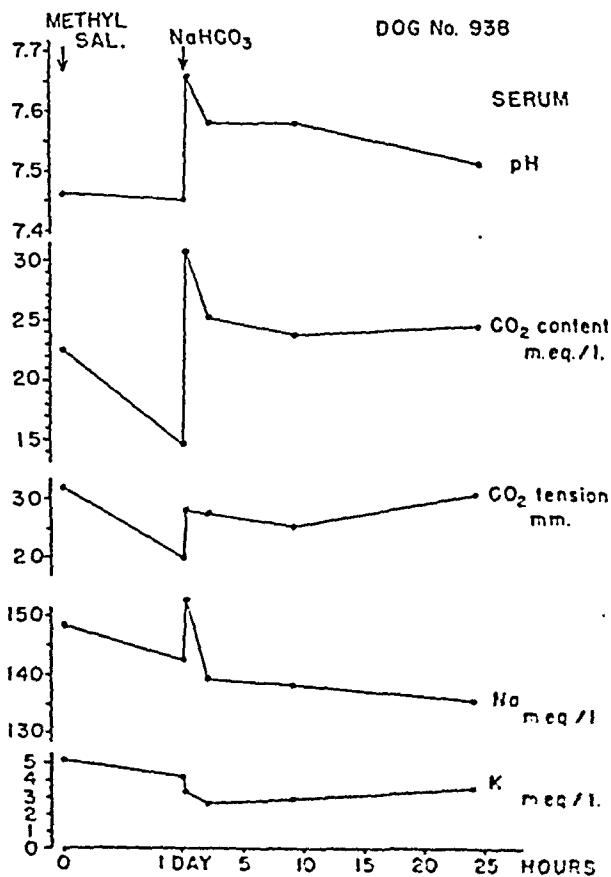


FIG. 8. THE EFFECT OF SODIUM BICARBONATE ON SALICYLISM

Dog 938, weighing 17 kgm., received 10 ml. of methyl salicylate. Twenty-four hours later, when hyperpnea led to lowered CO₂ content and tension of 15-20 mm., 10 grams of sodium bicarbonate were injected intravenously. The animal's respiration appeared unaffected. It continued the rest of the day, but the dog appeared normal in all respects 24 hours after the injection of the sodium bicarbonate.

normal human subjects. These findings also support the theory that salicylates exert their effect centrally and that a state of respiratory alkalosis characterizes salicylism. Sodium bicarbonate appeared to hasten the recovery from the intoxication, the dogs appearing normal within 24 hours after the administration of salicylates while untreated control animals appeared ill for 2 additional days. Sodium bicarbonate may exert its beneficial effect on the duration of the intoxication by causing increased urinary elimination of salicylate (8). The occurrence of tetany after the injection of sodium bicarbonate appears hardly surprising in view of the extent of the elevation of the pH produced. The possibility of tetany should be considered when the administration of sodium bicarbonate is contemplated in the treatment of salicylism in man.

DISCUSSION

The finding that salicylates cause a respiratory alkalosis does not in itself imply that these drugs exert their effect directly on the respiratory center. The possibility of a reflex mechanism for the hyperpnea by way of stimulation of the chemoreceptors in the carotid bodies must be considered. The evidence for the site of action is meager at the present time. Results have been obtained in 1 cat (20) which appeared to indicate that sodium salicylate exerted mainly a central effect, but a reflex component in the causation of the hyperpnea could not be excluded. The fact that the hyperventilation in the experiments here reported could be abolished, at least transiently by barbiturates, would also appear to indicate a direct action of salicylates on the respiratory center. As further indirect evidence of a direct influence on the brain may be cited the presence of other symptoms of irritation of the central nervous system, as well as the pathologic findings of congestion and petechial hemorrhages in the brain tissue.

The findings here reported deal only with humoral phases of the varied and widespread effects of salicylates. These drugs appear to act on the liver in a specific manner, affecting its content of glycogen and glutathione (21) and influencing the production of prothrombin (22, 23) and of fibrinogen (unpublished data). The effect of salicylates on the kidneys has been mentioned earlier. Alterations in the function of these organs may in

turn affect the electrolyte balance of the blood, modifying the usual picture of respiratory alkalosis.

Salicylates influence the intermediary metabolism in a variety of ways, affecting the excretion of nitrogen and of uric acid and inhibiting the activity of several oxidative enzyme systems (21). Their effect on the intermediary metabolism of the cells may underlie their physiologic actions; *e.g.*, the stimulation of respiration may result from an altered metabolism of the cells of the respiratory center.

CONCLUSIONS

1. The administration of methyl and sodium salicylates to monkeys and dogs causes primary hyperventilation with lowering of the CO_2 tension in the blood, leading to an alkalotic tendency, which may or may not be accompanied by decreases of bicarbonate. Frequently, lowered total electrolyte concentrations were observed. Low concentrations of inorganic phosphorus and of potassium were found.

2. The effect of pentobarbital sodium, barbitol sodium, paraldehyde and morphine on the hyperpnea of salicylism were studied in dogs. Hyperventilation could be suppressed and normal values for pH and CO_2 restored by the administration of pentobarbital sodium. All hypnotic drugs studied appeared to increase the toxicity of the salicylates. Morphine was found to exert a convulsant action without notable decrease of the hyperpnea.

3. The effect of sodium bicarbonate on the electrolyte structure of the blood in salicylism in dogs was to increase pH and bicarbonate content without affecting the tension of CO_2 . The administration of sodium bicarbonate appeared generally to shorten the duration of the salicylate intoxication, but in 1 animal fatal tetany occurred.

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THE EFFECT OF SALICYLATES ON THE ELECTROLYTE STRUCTURE OF THE BLOOD PLASMA.¹ II. THE ACTION OF THERAPEUTIC DOSES OF SODIUM SALICYLATE AND OF ACETYLSALICYLIC ACID IN MAN

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While hyperpnea and reduction of the CO_2 content of the blood are generally recognized findings of salicylate intoxication, little is known concerning the effect of therapeutic doses of salicylates. The prevailing impression (1) appears to be that such doses have no effect on the respiration. Investigations dealing with this question in experiments of a few hours duration showed small decreases of the CO_2 tension after single doses of salicylate (2, 3).

As part of an inquiry into the physiologic actions of salicylates, a study was undertaken on the changes in the electrolyte equilibrium of the blood of patients receiving therapeutic doses of salicylates.

METHODS

With precautions to avoid stasis, venous blood samples were drawn by means of a tightly-fitting syringe and needle and were delivered under paraffin oil. Determinations of serum pH, CO_2 , chloride, and sodium were carried out by methods previously described (4).

RESULTS

In Table I are presented the data on the electrolyte equilibrium of 21 rheumatic patients, 3 to 15 years old, and weighing 15 to 65 kgm., who had received sodium salicylate or acetylsalicylic acid orally in amounts ranging from 0.07 to 0.28 gram per kgm. body weight daily. The period of medication varied from 1 to 28 days. Fourteen of the patients developed one or more of the manifestations of salicylism. These varied in severity from a simple hyperpnea, the mildest symptom, to intense vomiting with hematemesis, which was observed in 2 of the patients. A relationship between dosage and symptoms is clearly indicated:

¹ A preliminary report was presented before the American Pediatric Society, May 1, 1942 (*Am. J. Dis. Child.*, 1942, 64, 200).

only 2 of 8 patients who received salicylates in doses of 0.15 gram per kgm. or less developed the signs of salicylism that appeared in 12 of 13 patients who received higher doses; a statistically highly significant difference. The pH values in the control sample of blood, i.e., samples that were taken either before the start of the treatment or several days after its discontinuance, ranged from 7.37 to 7.52, average 7.43. During the period of treatment, most patients showed small variations, mostly increases, in the pH of their blood, the highest value observed being 7.57. The CO_2 content, averaging 26.2 m.eq. per liter for the control samples, decreased in every instance and reached values of less than 20 m.eq. per liter in 14, and of less than 15 m.eq. per liter in 3 of the patients. The CO_2 tension, at a mean value of 42 mm. Hg in the control samples, also fell during each period of medication. In 13 of the patients, the tension reached values of below 30 mm. Hg and in 3, values of below 20 mm. Hg. The serum chloride averaged 102 m.eq. per liter in the control samples. It was below 100 m.eq. per liter before treatment started in 3 patients with cardiac decompensation. On 2 of these, the sodium also was determined and found to be 135 and 136 m.eq. per liter. In 18 of the patients the chloride increased, the maximum increase being 16 m.eq. per liter. The serum sodium, 140 m.eq. per liter in the control samples, during the period of medication deviated from the normal by less than 4 m.eq. per liter in 8 patients, decreased by as much as 9 m.eq. per liter in 4, and increased in 1 patient. This increase occurred in 1 of the patients with cardiac decompensation in whom a low serum sodium and chloride had been found in the preliminary blood sample.

In Figures 1 to 3 are portrayed different patterns of change in the electrolyte equilibrium of the plasma, representing diverse types of adjust-

ment to the effect of salicylates. In Figure 1 are shown the data for the blood of F. S. who received 0.20 gram per kgm. of acetylsalicylic acid daily for 5 days. During this time, the pH increased slightly, the CO_2 content and tension dropped, and the chloride content increased to an extent corresponding to the loss of the bicarbonate. The sodium concentration, not shown in the figure, did not change notably. Withdrawal of the drug was followed by a return of the values to normal. In Figure 2 are presented the findings on the blood of A. D., who received the same dosage of acetylsalicylic acid for a period of 7 days. In this case, the decrease of the CO_2 content was only partly

counter-balanced by the change in the chloride value; instead, the sodium content decreased to 134 m.eq. per liter. Another type of response to salicylate administration is shown in Figure 3. Patient B. S., who received 0.23 gram per kgm. of sodium salicylate for 6 days, showed a transient elevation of the pH to a value of 7.57 with a small decrease of the CO_2 content and a marked diminution of the CO_2 tension. In the latter part of the period of medication the pH returned to normal, and the CO_2 content decreased to a low level, without much further change in the CO_2 tension. The sodium content of the serum of this patient was also significantly decreased, while the chlo-

TABLE I
The effects of salicylate medication on the plasma electrolytes

Case number	Age	Weight	Period of medication*	Salicyl dosage	pH	CO ₂	pCO ₂	Chloride	Sodium	Symptoms of salicylism
	years	kgm.	days	grams per kgm. per day		m. eq. per liter	mm. Hg	m. eq. per liter	m. eq. per liter	
1	15	43	before	0	7.44	30.8	46	100	142	
			7	S 0.07	7.47	23.2	32	106	138	
			5 after	0	7.41	29.4	41	104	141	None
2	12	65	before	0	7.43	29.0	44	96	135	
			5	S 0.09	7.41	22.5	35	107	146	None
			8	S 0.09	7.45	21.5	31	108	140	None
			5 after	0	7.46	28.3	41	99	134	
			5	S 0.09	7.47	25.4	35	105	144	None
			8	S 0.09	7.46	19.9	29	107	144	None
3	9	33	before	0	7.48	29.7	41	101	141	
			2	S 0.12	7.51	26.2	33	105	142	None
			4	S 0.12	7.48	25.6	34	107	141	None
			1 after	0	7.48	27.3	38	106	140	
4	13	47	before	0	7.48	30.8	42	100	143	
			4	S 0.13	7.45	24.5	37	107	145	None
			11	S 0.13	7.46	21.6	30	108	141	None
5	12	45	before	0	7.42	29.7	45	105	141	
			9	S 0.13	7.45	26.6	38	107	135	None
6	14	41	before	0		24.8		104		
			4	S 0.15	7.43	17.7	24	104		Vomited once, lethargic
7	12	48	before	0	7.52	31.0	37	95	136	
			4	S 0.17	7.44	23.1	34	102	139	None
			21	S 0.17	7.44	19.4	28	111	139	None
8	9	31	28	S 0.20	7.49	13.5	17	112		Dyspnoic, hematemesis
9	5	17	before	0	7.43	25.8	39		140	
			2	S 0.23	7.57	24.8	27			Vomiting, hiccups
			6	S 0.23	7.41	14.4	22		133	Vomiting, hiccups
			3 after	0	7.41	24.7	39		136	
10	8	23	before	0	7.39	29.2	49	109	139	
			1	S 0.26	7.42	24.8	37	103	143	Vomiting
11	11	23	before	0	7.45	28.0	43	101	139	
			2	S 0.26	7.47	16.1	22	108	132	Vomiting
			5 after	0	7.46	27.9	40	101	141	
			5	S 0.09	7.47	23.9	33	107	141	None

TABLE I—Continued

Case number	Age	Weight	Period of medication*	Salicyl dosage	pH	CO ₂	pCO ₂	Chloride	Sodium	Symptoms of salicylism
	years	kgm.	days	grams per kgm. per day		mm. eq. per liter	mm. Hg	mm. eq. per liter	mm. eq. per liter	
12	3	15	2	S 0.27	7.52	14.6	18	114	138	Hyperpneic
			3 after	0	7.44	26.6	40	108	138	
13	9	21	before	0		26.5		97		Vomiting
			4	S 0.28	7.52	18.1	22	98		
14	12	33	before	0	7.45	27.2	40	102		None Vomiting, irritable
			2	A 0.12	7.40	23.4	38	101		
			4	A 0.12	7.41	19.7	30	109		
15	13	52	before	0	7.42	27.0	42	102		Listless, ringing in ears Listless, ringing in ears
			2	A 0.12	7.42	17.4	27	110		
			4	A 0.12	7.46	22.3	31	106		
			3 after	0	7.40	25.4	42	105		
16	14	41	6	A 0.18	7.41	19.7	31	106	135	Hyperpneic Vomiting, hematemesis
			12	A 0.18	7.49	21.0	27	102	134	
			20 after	0	7.44	25.8	38	105	140	
17	13	32	2	A 0.19	7.49	25.2	34	105	135	None Vomiting
			4	A 0.19	7.43	22.3	33	104	135	
18	6	21	before	0	7.37	24.9	42	109	140	None Vomiting, lethargic
			2	A 0.20	7.46	17.2	24	112	142	
			4	A 0.20	7.44	12.6	18	112	139	
19	13	30	5	A 0.20	7.50	20.1	25	110	134	Hyperpneic Hyperpneic
			7	A 0.20	7.48	16.4	21	110	134	
			3 after	0	7.38	26.0	44	106	143	
20	12	31	before	0	7.42	29.6	47	104	143	None Vomiting
			3	A 0.20	7.45	19.5	28	111	141	
			5	A 0.20	7.46	19.4	27	114	146	
			1 after	0	7.40	20.2	32	112		
			2 after	0	7.44	25.8	38	108	141	
			4 after	0	7.51	29.1	38	104		
21	6	24	3	A 0.21	7.38	15.3	24	112		Dyspneic, irritable
			3 after	0	7.37	26.6	47	105		

S Sodium salicylate.

A Acetylsalicylic acid.

* Samples designated "before" were taken before salicylate administration was begun. The figures followed by "after" indicate the period after medication had been stopped.

ride, not shown, changed little. In 11 of 13 patients, on whom repeat-samples of blood were obtained during periods of salicylate medication, further decreases of the CO₂ tension, accompanied by diminished CO₂ content, were observed. The mean difference between the CO₂ tensions of the first and the second samples during periods of medication, statistically highly significant, was 3.6 mm. Hg with a S.E. of 0.9 mm. Hg. This indicates a cumulative effect of the drug.

No marked difference was observed between the effect of sodium salicylate and of acetylsalicylic acid; however, the data are insufficient for a detailed comparison.

In Figure 4 is presented a scatter diagram and

the calculated regression line depicting the relationship between the CO₂ tension and the daily dosage of salicylate. All CO₂ tension data listed in Table I are plotted, with the exception of that of case 10 which was obtained within 1 day of the start of medication. The relationship between salicylate dosage and CO₂ tension, evident to cursory inspection, is highly significant. It is of interest to note that the regression line cuts the ordinate at a CO₂ tension value of 40.4 mm. Hg, close to the mean value of 42 mm. Hg of the control samples. The equation for the regression line is:

$$y = 40.4 + (-72.3) x_1$$

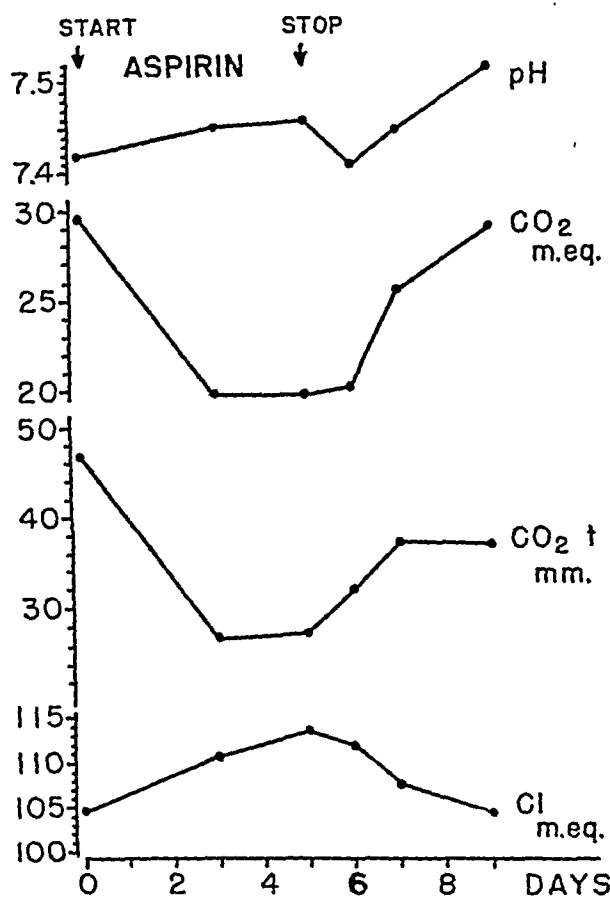


FIG. 1. DATA FOR THE BLOOD OF F. S.

where y indicates the CO₂ tension in mm. Hg, and x_1 the daily dose of salicylate in grams per kgm. bodyweight.

If the period of medication is taken into account, a closer fitting regression equation is obtained:

$$y = 42.2 + (-73.7) x_1 + (-0.27) x_2$$

where y and x_1 have the same meaning as above, and x_2 indicates the period of medication in days. Both regression coefficients are significant as tested by the t test, t_1 being 6.06, and t_2 being 2.02.

This result, based on all observations, reinforces the conclusion reached from the analysis of the repeat-samples alone and indicates that the effect of salicylate increases with the period of medication.

DISCUSSION

Strict comparison of the data of the present study, obtained on venous blood, with the results

on arterial plasma of the preceding investigation cannot be undertaken owing to variations of the average arterio-venous difference and of the composition of the blood in different veins. On the average, the pH of venous plasma is about 0.03 units lower, and the CO₂ content and tension about 2 m.eq. per liter and 5 mm. Hg higher, respectively, than are the corresponding values for arterial plasma. Estimates of the composition of arterial plasma by the use of such correction factors are subject to errors in opposite directions; veno-stasis would tend to increase the arterio-venous differences, while increased cardiac output would lead to an approach of arterial and venous values. Under the conditions of the present study, in all likelihood the first influence predominated, leading to an underestimate of the effect of salicylates on the electrolyte equilibrium of the blood.

The data on human subjects tend to bear out the conclusions reached from animal experiments; namely, that salicylates produce a primary hyperventilation with consequent decrease of the CO₂ tension and a tendency toward increased pH values. In the human subjects, who received

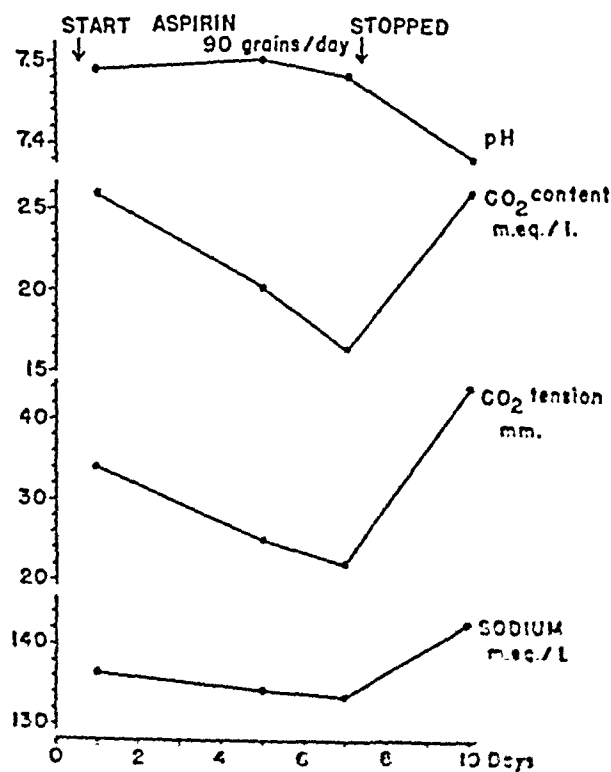


FIG. 2. DATA FOR THE BLOOD OF A. D.

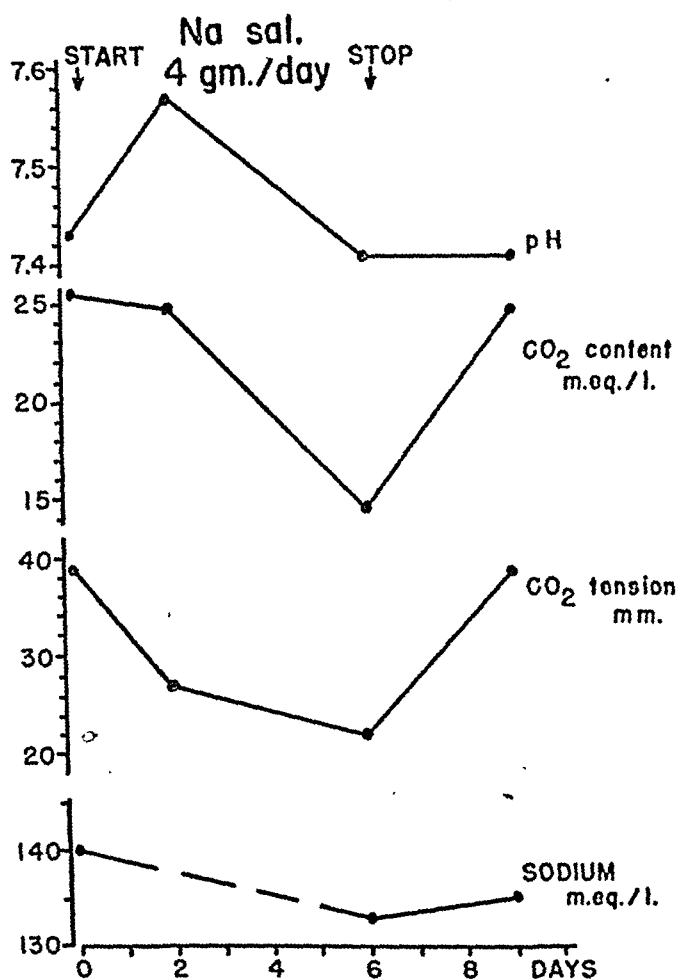


FIG. 3. DIFFERENT PATTERNS OF CHANGE IN THE ELECTROLYTE EQUILIBRIUM OF THE PLASMA

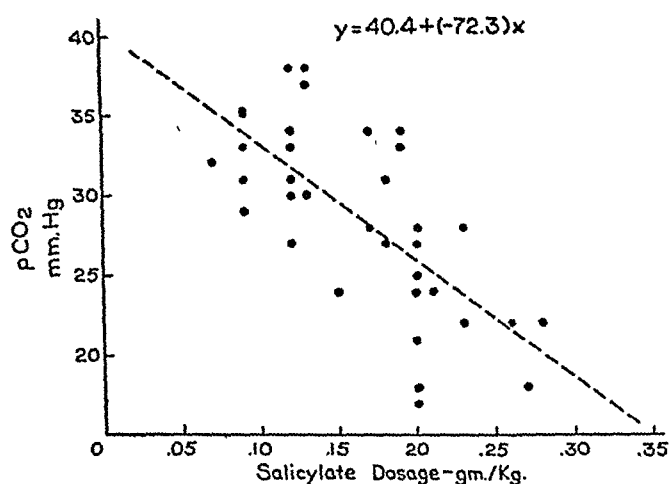


FIG. 4. RELATIONSHIP BETWEEN THE CO_2 TENSION AND THE DAILY DOSAGE OF SALICYLATE

smaller doses than the experimental animals, pH change was usually slight, indicating successful compensation. The compensatory decrease of the bicarbonate concentration in most cases was offset

by a commensurate increase of chloride so that the total electrolyte concentration of the plasma was maintained. In 4 of the patients, however, decreases of the serum sodium were seen such as were commonly observed in dogs with salicyl poisoning.

A decreased CO_2 tension, found in every patient studied, may be taken as *prima facie* evidence of increased pulmonary ventilation; however, hyperpnea was not always noticed. This may have been because salicylates at first affect the amplitude, rather than the frequency of the respirations.

The fact that small doses of salicylates cause chemical changes of the blood in the same direction as those observed in severe poisoning, may indicate that, in these respects at least, the normal pharmacologic and toxic effects of salicylates differ in degree only.

SUMMARY

In the plasma of patients receiving therapeutic doses of sodium salicylate or of acetylsalicylic acid, decreased tension and content of CO_2 , and small changes of pH were found. The plasma chloride was usually elevated, and the sodium was occasionally decreased. The effect of salicylate appeared to be proportional to the dose administered and to increase with the period of medication. The results indicate that salicylates produce primary hyperpnea with consequent decrease of CO_2 tension. Various types of adjustment of the electrolyte equilibrium to the hyperventilation were observed.

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EPIDEMIC OF ACUTE RESPIRATORY DISEASE ASSOCIATED WITH ATYPICAL PNEUMONIA

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Recently, the concept has developed that atypical pneumonia is merely an incident in what is usually a mild epidemic disease of the respiratory tract (1 to 6). A group of workers (3) summarize this idea:

"There is thus no sharp dividing line, other than roentgen evidence . . . to differentiate cases of atypical pneumonia from cases of acute bronchitis resembling atypical pneumonia and from cases of other illness of the respiratory tract. . . . It seems probable that at least some of these minor illnesses may be milder infections of the same specific nature as atypical pneumonia. Proof of such a hypothesis depends on the demonstration of the causative agent."

Although numerous clinical and epidemiological descriptions (7 to 20) of the disease have appeared, etiological studies have been largely disappointing. A few of the cases, especially the more severe ones, have been demonstrated to be caused by ornithosis (21) and related agents, "Q" Fever (22) and lymphocytic choriomeningitis (23) but it was recently concluded that "a

virus responsible for a large proportion of the cases of so-called virus pneumonia has not been satisfactorily established (24)."

The present communication deals with an explosive outbreak of respiratory disease, associated with atypical pneumonia, the etiology of which has been indicated to be the virus described by certain investigators (25).

The epidemic started in the only grade school of Kasson, a town in southeastern Minnesota, the population of which in 1940 was 1,230 people and spread into households of the school children. There were a total of 191 cases.

Table I shows the symptomatology of the disease, with cases divided arbitrarily into 3 groups: "severe," those with fever and bed-rest of more than 3 days; "moderate," those with fever and bed-rest of 3 days or less; and "mild," those without fever or whose temperature was not taken and bed-rest of 3 days or less. The "severe" cases of this outbreak would actually correspond to a fairly mild form of the disease as commonly described; none were hospitalized and only 29 per cent of the "severe" cases were in bed longer than 2 weeks, none longer than 3 weeks.

TABLE I
Symptomatology

	"Severe" cases		"Moderate" cases		"Mild" cases		Total cases	
	number	per cent	number	per cent	number	per cent	number	per cent
No. of cases	31	100	47	100	113	100	191	100
Fever	31	100	47	100			78	41
Cough	31	100	37	79	74	65	142	74
Headache	25	81	21	45	14	12	60	31
Chest signs*	12	39					12	6
Sore throat	1	3	10	21	17	15	28	15
Coryza	1	3	12	26	54	42	67	35
Chest pain	2	6	1	2	1	1	4	2
Chilliness	6	19	3	6	1	1	10	5
G.I. symptoms	7	23	6	13	5	4	18	9
Days in bed								
1 to 3			28	60	9	8	37	19
4 to 7	12	39					12	6
8 to 14	10	32					10	5
15 to 21	9	29					9	5

* Only 26 patients examined during the acute stage of the illness.

Of the 191 cases, 26 received chest examinations by a physician during the acute stage of the illness, these being the more seriously affected. Râles or dullness was noted in 12 of the 26 patients examined. The remainder of the cases did not of their own initiative consult a physician, usually because of the mildness of the symptoms. Information concerning these was gathered through interview of the children and their parents during the acute stage or within a few days thereafter by a public health physician and public health nurses. Only 7 of the more severe cases were examined by x-ray, usually during early convalescence, and evidence of pneumonia was present in 5.

TABLE II
Attack rate in grade school

	Number in room	Number ill	Attack rate <i>per cent</i>
First	23	9	39
Second	19	11	58
Third	19	10	53
Fourth and fifth	35	30	86
Fifth and sixth	36	22	61
Seventh	29	17	59
Eighth	25	18	72
Totals	186	117	63

Cough was the most common symptom, being present in 74 per cent of all cases; it was hacking in nature with little or no sputum and almost always persisted even in mild cases for 10 days or longer. This characteristic, persistent cough, to-

gether with the relative infrequency of coryza, served in general to distinguish the "mild" cases of the outbreak clinically from the common cold and to identify them with the more severe cases. It will be noted that the incidence of coryza and sore throat was somewhat higher in the milder cases than in the severe, and the incidence of cough lower. Some of these milder cases may have been common colds or minor bacterial infections. However, no dividing line would be found clinically or temporally between the mild and severe cases of the epidemic.

The attack rates in the various rooms of the grade school are given in Table II. Of the 186 pupils in the school, 117 or 63 per cent were affected. The attack rates in the different rooms ranged from 39 to 86 per cent.

Household occurrence of the disease, by age groups, is shown in Tables III and IV. In the 94 households affected there were 329 persons exposed to secondary attack, considering the first case in each household as the primary attack. Ninety-seven or 29 per cent of the 329 persons exposed in households contracted the disease. However, most of these (56 of the 97) who did become ill were in the age group 5 to 14 years so that they were also exposed in school. Only 41 cases occurred among the 230 persons who were exposed in households but not in the school, giving a corrected secondary household attack rate of 18 per cent, to be compared with an attack rate of 63 per cent in the school. This difference may have been due to an age factor in

TABLE III
Age and sex distribution in primary and secondary cases

		Age years						Totals
		0 to 4	5 to 9	10 to 14	15 to 19	20 or more	?Age	
Primary cases:	Males	1	15	24	4	2	0	46
	Females	2	13	21	2	10	0	48
	Totals	3	28	45	6	12	0	94
Secondary cases:	Males	3	13	13	2	9	0	40
	Females	7	12	18	4	16	0	50
	Totals	10	25	31	6	25	0	90
Total cases:	Males	4	28	37	6	11	0	86
	Females	9	25	39	6	26	0	95
	Totals	13	53	76	12	37	0	181

ery alkalosis in monkeys and and methyl salicylate; the influ- drugs and of sodium bicarb- isoning. J. Clin. Invest.

TABLE IV
Secondary household attack rate*

		Age					
		0 to 4	5 to 9	10 to 14	15 to 19	20 or more	Age
Males	Number exposed Number of cases	years					
		8 3	17 13	22 13	9 2	93 9	8 0
	Attack rate (A.R.)	per cent					
		38	76	59	22	10	0
Females	Number exposed Number of cases	years					
		12 7	17 12	30 18	18 4	90 16	5 0
	Attack rate (A.R.)	per cent					
		58	71	60	22	18	0
Totals	Number exposed Number of cases	years					
		20 10	34 25	52 31	27 6	183 25	13 0
	Attack rate (A.R.)	per cent					
		50	73	60	22	14	0

* Higher rates in younger age groups, especially the 5 to 9 and 10 to 14 groups, may reflect spread within the school rather than differential age attack rates.

susceptibility or to some unknown factor favoring spread in the school.

Although dates of onset were found to extend from September 1 to November 2, 1942, as shown in Table V, over 90 per cent of the cases occurred from September 29 to October 26, and this latter probably represented the true epidemic period. Nearly half of the cases appeared during the week October 6 to 12. The outbreak may thus be said to have been explosive. In this respect, it differs from most of the epidemics which have been noted to extend over several months. One experience described (19) bears a close similarity to the present one.

The disease occurred indiscriminately among users of the one pasteurized milk supply, one public raw milk supply, and several private raw milk supplies, thereby eliminating milk from consideration as a possible source of infection. Water was excluded on similar grounds. Pigeons abounded in the school house and other nearby buildings. Investigation disclosed pigeons' droppings in the openings of the ventilating ducts into the school rooms, and 1 dead pigeon was found in the ventilating system. It was also stated that

live pigeons occasionally came through the ventilating system into the school rooms. In view of these facts, ornithosis was considered a possible etiology. Further investigation (detailed below) did not substantiate this hypothesis. The behavior of the disease in the households definitely suggested a respiratory mode of spread. The incubation period, estimated from the secondary occurrence in the household-exposed but non-school-exposed group, was from 8 to 21 days.

Throat washings taken early in the course of the disease from several typical cases were inocu-

TABLE V
Dates of onset

Dates	Number of cases	Percent of cases
September 1 to 7, 1942	2	1.2
September 8 to 14, 1942	1	0.6
September 15 to 21, 1942	3	1.6
September 22 to 28, 1942	5	3.0
September 29 to Oct. 5, 1942	28	16.6
October 6 to 12, 1942	72	46.6
October 13 to 19, 1942	32	18.9
October 20 to 26, 1942	16	9.3
October 27 to Nov. 2, 1942	3	1.6
Total	162	100.0
Date not stated	22	
Grand total	184	

lated² into mice. Pulmonary lesions occurred and some deaths after a few passages, but the responsible agent could not be differentiated from mouse pneumonia virus of Horsfall. It was not considered as having any etiological relation to the cases. Ten pairs of blood specimens showed no significant rise in titer between the acute-phase and convalescent specimens against influenza A (PR-8 strain) or influenza B (Lee strain).

The epidemiological evidence suggested ornithosis. Four pairs of blood specimens were submitted to Dr. K. F. Meyer, Hooper Foundation for Medical Research, San Francisco, California who reported that the complement-fixation test showed no increase in antibodies against psittacosis antigen. Ornithosis virus was isolated³ from the organs of 10 out of 35 pigeons captured at the school. However, no real connection between the

² This work was carried out at the Rockefeller Influenza Laboratory, Division of Preventable Diseases, Minnesota Department of Health, under the direction of Dr. Carl Eklund.

³ This work was carried out at the Rockefeller Influenza Laboratory, Division of Preventable Diseases, Minnesota Department of Health, under the direction of Dr. Carl Eklund.

human disease and the pigeon virus could be established. The fact that this virus can ordinarily be isolated from throat washings of patients and established in mice suggests that the epidemic was not due to ornithosis. Also if the epidemic had been due to ornithosis, a higher proportion of severe cases would have been expected.

Recently, a group of workers (25) have described a virus which they isolated from cases of atypical pneumonia. Specimens of sera which have been saved from cases of the Kasson epidemic were submitted to Dr. Monroe D. Eaton for neutralization test with the new virus. He reports that specimens of the sera showed a significant rise in antibody titer as shown in Table VI. Paired serum specimens from 7 cases were tested. In 5 of these cases a 4-fold increase in antibodies was demonstrated. In one case (P. S.) a 2-fold increase was found, and in the remaining case (J. B.) titers of both the acute-phase and convalescent serum specimens were above 64, suggesting recent infection with the virus of atypical pneumonia. Most of these cases were among the "severe" cases of the epidemic but were not severe in comparison with the usual accounts of the

TABLE VI
*Neutralization tests with the virus of atypical pneumonia
(strain De, amniotic passage)*

Patient	Sex	Age	Date of onset	Date of specimen	Serum dilutions*				Horse serum control**	Normal amniotic***	Test animal
					1:4	1:8	1:16	1:64			
B. B.	M	11	10/ 8/42	10/10/42 10/22/42	3/4	2/3 0/3	0/3		5/7	0/7	C.R.† C.R.
J. B.	F	35	10/20/42	10/23/42 11/17/42	0/4 0/4		0/4 0/4	0/4 0/4	2/4	0/4	C.R. C.R.
D. K.	F	9	10/10/42	10/12/42 11/ 3/42	2/2 0/3	2/3	0/6	0/3	3/6	0/3	C.R. C.R.
J. A.	M	14	10/ 7/42	10/10/42 12/ 7/42	3/4 0/4		0/4		3/4	0/4	C.R. C.R.
R. E.	M	13	10/11/42	10/12/42 11/ 3/42	2/4 0/4		2/3 0/4		2/4	0/4	C.R. C.R.
E. S.	M	35	11/ 1/42	11/ 3/42 11/17/42	2/4 0/4	0/4	2/4		7/8	0/8	H.† H.
P. S.	F	11	10/11/42	10/12/42 11/ 3/42	4/4 0/4	2/4	4/4				H. H.
J. W.	M	10	10/ 6/42	10/10/42 11/ 3/42	3/4 0/4		0/4		3/4	0/4	H. H.

* Numerator of fraction = number of animals with pulmonary lesions; denominator = number of animals tested.

** Control for virus activity.

*** Control for intercurrent respiratory infection in animals.

† C.R. = Cotton rat.

‡ H. = Hamster.

disease. Although no sera from the extremely mild cases (those who were not in bed at all) were tested, it seems reasonable to assume from the clinical and temporal similarity of the cases that only one agent was involved in the outbreak.

DISCUSSION

The neutralization test thus indicates that the virus described by a group of investigators (25) was responsible for the Kasson epidemic. Further study of the virus should elucidate its relationship to that group of cases among the minor respiratory diseases characterized by a lingering cough.

Present evidence suggests that a disease entity is being established as a segment of the common respiratory diseases to take its place along side the common cold and influenza. As was pointed out as early as 1940 (1), the disease is probably not primarily a pneumonia, nor in view of the work accomplished (25) can it any longer be said that its etiology is unknown. The designation "atypical pneumonia" seems to exaggerate what is probably only an incident in the disease. Before this concept can be considered established, it will be necessary to isolate the virus from cases in which thorough clinical study excludes pneumonia, demonstrate its etiological relationship to the cases by neutralization test, and finally show its correspondence to virus isolated from cases with atypical pneumonia.

The author is grateful to Dr. Monroe D. Eaton, California State Department of Health, Dr. Carl Eklund, Minnesota State Department of Health, Dr. K. F. Meyer, Hooper Foundation, and Dr. F. R. Heilman, Rochester, Minnesota, for the work mentioned in the paper and for valuable advice and encouragement. Dr. D. E. Affeldt, Health Officer, Kasson, Minnesota, greatly assisted the field study of the outbreak, and Mr. I. J. Fox, Superintendent, Kasson Public Schools, gave excellent co-operation.

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THE EFFECT OF ALTERATIONS IN BLOOD VOLUME ON THE ANEMIA AND HYPOPROTEINEMIA OF HUMAN MALARIA¹

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There is general agreement that humans with active malarial infections⁴ develop anemia and hypoproteinemia. The anemia is usually considered to be secondary to erythrocyte destruction by the intracellular parasites, but no adequate explanation has been offered for the hypoproteinemia. Since laboratory techniques commonly used measure only the relative amounts of chemical and cellular components of the blood, it cannot be assumed that these relative measurements always reflect fluctuations in the total amounts of these constituents.

In order to determine alterations in the total amounts of circulating plasma proteins and red blood cells, this study of the blood volume changes associated with clinically active, untreated malaria was undertaken. Simultaneous determinations of the plasma proteins, plasma bilirubin, and erythrocyte concentrations were made so that the relative and total amounts of these elements might be calculated and compared.

METHODS AND MATERIALS

Nine patients with central nervous system syphilis, but otherwise relatively normal, served as experimental subjects. The group consisted of 5 white and 3 negro males and 1 negro female. Four patients were inoculated by the bites of *Anopheles quadrimaculatus* infected with *Plasmodium vivax* (McCoy strain), 1 patient by the intravenous administration of trophozoites of *P. vivax* (McCoy strain), and 4 patients by the intravenous injection of trophozoites of *P. falciparum* (Costa strain). These data are summarized in Table I.

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Tennessee.

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⁴ For the purpose of this report, the period of active malaria is considered to be the period of visible parasitemia. Consequently, the days of disease are the days of parasitemia, while days which precede or follow the days of parasitemia are identified by minus or plus signs respectively.

Parasite counts were performed daily on the peripheral blood (1).

Plasma volume was measured (in fasting subjects) with the dye T-1824 (2). However, it was suggested (3) that a single 10-minute sample of blood be used in place of an extrapolation curve to calculate the degree of dye dilution and this was done, except that we employed a single 15-minute sample. Patients were given 5 ml. of a 0.5 per cent solution of the dye⁵ for each measurement and the absorption coefficient of the dyed plasma was then determined with a Beckman photoelectric spectrophotometer.

TABLE I

Summary of descriptive data concerning 9 malaria patients studied by the T-1824 blood volume method

Patient	Sex	Color	Age	Species	Method of inoculation
1	M	W	29	<i>P. vivax</i>	Trophozoites
2	M	C	35	<i>P. falciparum</i>	Trophozoites
3	M	C	43	<i>P. falciparum</i>	Trophozoites
4	M	W	39	<i>P. vivax</i>	Sporozoites
5	M	W	41	<i>P. vivax</i>	Sporozoites
6	M	W	34	<i>P. vivax</i>	Sporozoites
7	F	C	36	<i>P. falciparum</i>	Trophozoites
8	M	W	48	<i>P. vivax</i>	Sporozoites
9	M	C	43	<i>P. falciparum</i>	Trophozoites

All blood samples were heparinized and, in addition to dye concentration, were analyzed for plasma protein concentration (4), for plasma bilirubin (5), and for red blood cell concentration by means of the hematocrit index. Hematocrits were done in duplicate and centrifuged for 30 minutes at 3,000 r.p.m. in Wintrobe tubes.

White patients who had plasma volumes determined on consecutive or nearly consecutive days became visibly discolored, and this served as a limiting factor for the frequency with which determinations could be made in the same individual. This "blueing" of the skin gradually disappeared over a period of from several days to a few weeks.

Each patient had one or more "base-line" plasma volume measurements during the period prior to the clinical expression of malaria. Further plasma volume determinations were made during both febrile and afebrile phases of clinical activity and, whenever possible, during convalescence.

⁵ Obtained from the William R. Warner Company of New York, N. Y., in ampoules of this size.

TABLE II
Summary of blood volume and other studies in 9 patients with vivax and falciparum malaria¹

Patient	Day of disease	Total hours of preceding fever	Fever	Total blood volume		Plasma volume		Erythrocyte mass		Venous hematocrit		Plasma proteins		Plasma bilirubin		Transfusions ²	
				ml.	per cent	ml.	per cent	ml.	per cent	Index	Change	grams	per cent	mgm. per 100 ml.	Total	Day of disease	Material
1	-1	0	no	6206		3662		2544		41.0		7.00		0.23	8.4		
	1	41.5	no	6580	+5	4061	+11	2449	-4	38.0	-7	6.86	+9	0.99	40.2	11	RBC
	11	41.0	no	6940	+12	4393	+10	2537	0	37.5	-9	6.35	+9	2.49	109.4		
	14	59.0	no	6788	+9	4413	+21	2375	-7	35.0	-14	6.36	+10	0.99	47.7		
	4-25	69.5	no	5937	-4	3978	-10	1959	-23	33.0	-20	6.52	+1	0.46	18.3	+2	Whole Blood
2	4-25	69.5	no	5623	-8	3300	-10	2393	-6	41.0	0	7.30	+6	0.17	5.6		
	-4	0	no	5105		3027		2378		44.0		7.35		0.35	10.6		
	20.0	105°F.	no	5057	-6	3186	+5	1871	-21	37.0	-16	6.78	-3	4.58	145.9	10	RBC
	16.0	101°F.	no	5235	-57	1628	-46	697	-71	30.0	-32	5.97	-56	1.91	36.1	10	Plasma
	11	36.5	no	5251	-3	3836	+27	1418	-40	27.0	-39	6.07	+4	0.52	19.9	13	Whole Bl.
3	-6	0	no	6141		3387		2754		46.5		7.52		0.70	27.4		
	4	47.0	no	6076	-2	3435	+1	2591	-6	43.0	-8	7.18	-5	1.57	53.9		
	11	91.0	no	6560	+7	4166	+23	2394	-13	36.5	-22	7.00	+12	0.46	19.2		
	21	91.0	no	6110	0	3930	+16	2210	-20	36.0	-23	8.03	+21	0.35	13.8		
	-1	0	no	5862		3459		2403		41.0		6.78		0.75	32.3	15	RBC
4	12	25.5	no	6281	+7	4305	+24	1979	-22	32.0	-22	6.78		0.64	25.2		
	15	25.5	no	5832	0	3193	+16	1837	-24	31.5	-23	8.00		0.12	3.2		
	4-1	25.5	no	6312	+8	3945	+14	2367	-2	37.5	-9	7.63	+10	0.58	21.6		
	-1	0	no	5215		2660		2555		49.0		5.98		0.23	8.6		
	11	49.5	no	6210	+19	3726	+40	2183	-3	40.0	-18	7.00	+29	0.29	7.1		
5	4-9	96.0	no	5394	+3	3722	+40	1672	-35	31.0	-37	6.83	-17	0.46	14.0		
	4-6	96.0	no	4384	-15	2455	-7	1929	-24	44.0	-10	7.00	0	0.41	15.0	15	RBC
	-14	0	no	5306		3051		2255		42.5		5.90	+4	0.17	6.5	17	RBC
	11	52.5	no	5742	+8	3650	+20	2050	-9	36.0	-15	5.60		0.81	18.2		
	4-22	90.5	no	5964	+7	3027	+30	1935	-14	39.0	-8	7.85	-11	0.70	18.3	16	RBC
6	1	0	no	3621		2209		1412		39.0		7.85		0.29	9.6		
	11	48.5	no	3340	-8	2241	+1	1079	-24	32.5	-17	6.85	-5	0.99	35.8		
	11	96.5	no	3649	+10	2441	+18	1026	-27	28.2	-27	6.60	+5	1.16	46.0		
	17	96.5	no	4177	+15	2612	+19	1545	+9	37.0	-9	6.69	+5	0.46	14.5		
	27	95.5	no	3601	-5	2306	+4	1297	-12	36.0	-8	8.55	+5	0.29	9.4		
7	-5	0	no	5708		3197		2511		44.0		7.01		0.29	9.6		
	9	10.0	no	5517	-3	3090	-3	2127	-3	41.0	0	6.92	-5	0.99	35.8		
	17	22.0	no	6284	+10	3962	+24	2326	-7	37.0	-16	5.90	+5	1.16	46.0		
	21	11.0	no	4447	-15	3155	-1	1698	-32	35.0	-20	7.10	+5	0.46	14.5		
	4-14	11.0	no	5126	-5	3256	+2	2170	-14	40.0	-9	7.20	+5	0.29	9.4		
8	-4	0	no	5521		3175		2316		42.5		7.52		0.41	13.0		
	10	46.0	no	4958	-12	3187	0	1678	-15	31.5	-19	7.22	-4	1.39	44.2		
	23	62.5	no	4576	-17	3369	+6	1207	-49	26.5	-37	7.00	-1	0.58	19.5		
	41	62.5	no	4622	-17	2935	+8	1637	-28	36.5	-14	7.90	+10	0.23	6.8		

¹ The first series of figures for each patient represents the control values. Per cent change represents deviation from the control value.
² Measurements preceded transfusions where both are recorded on the same day.

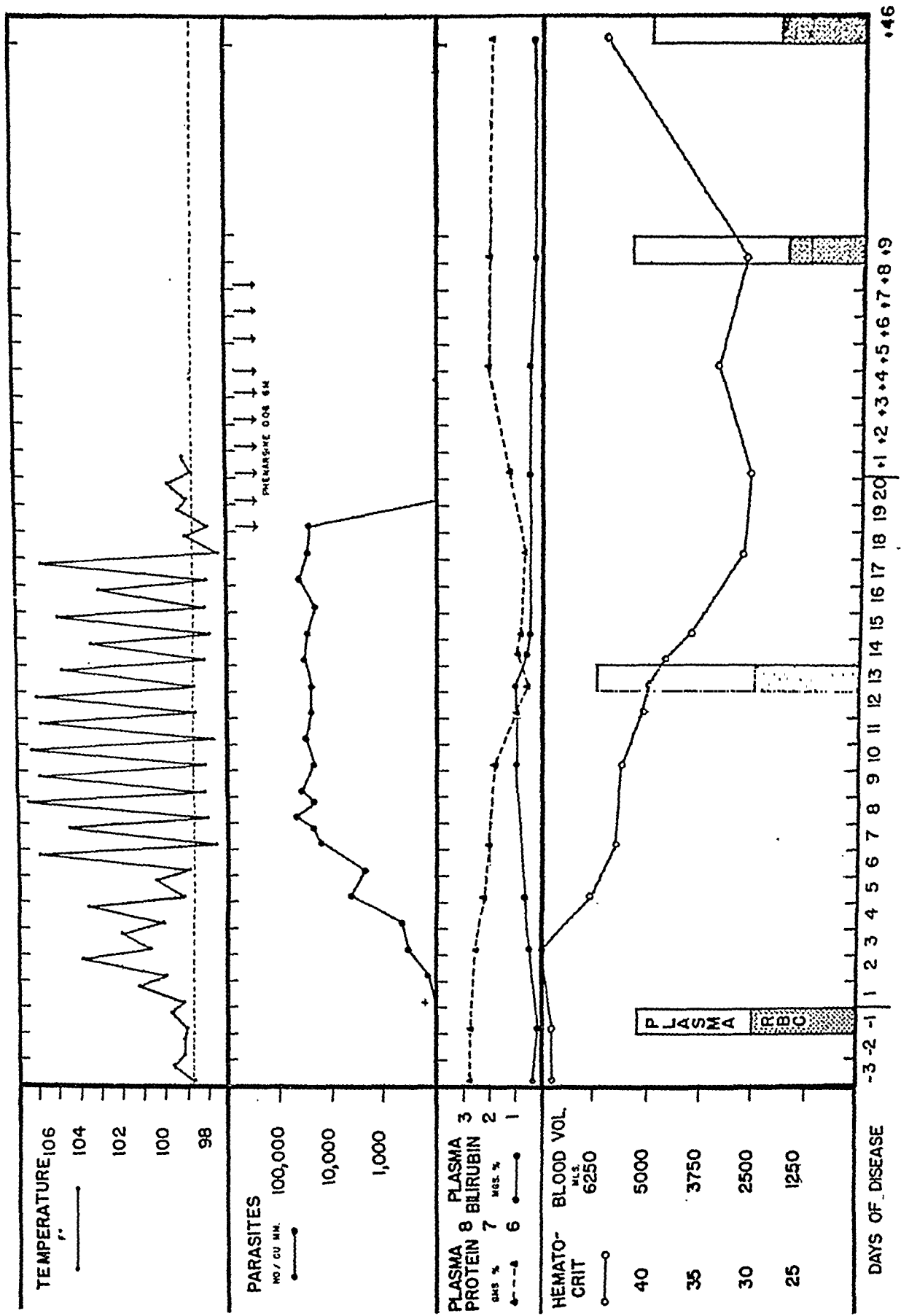


FIG. 1. COURSE OF PATIENT 5

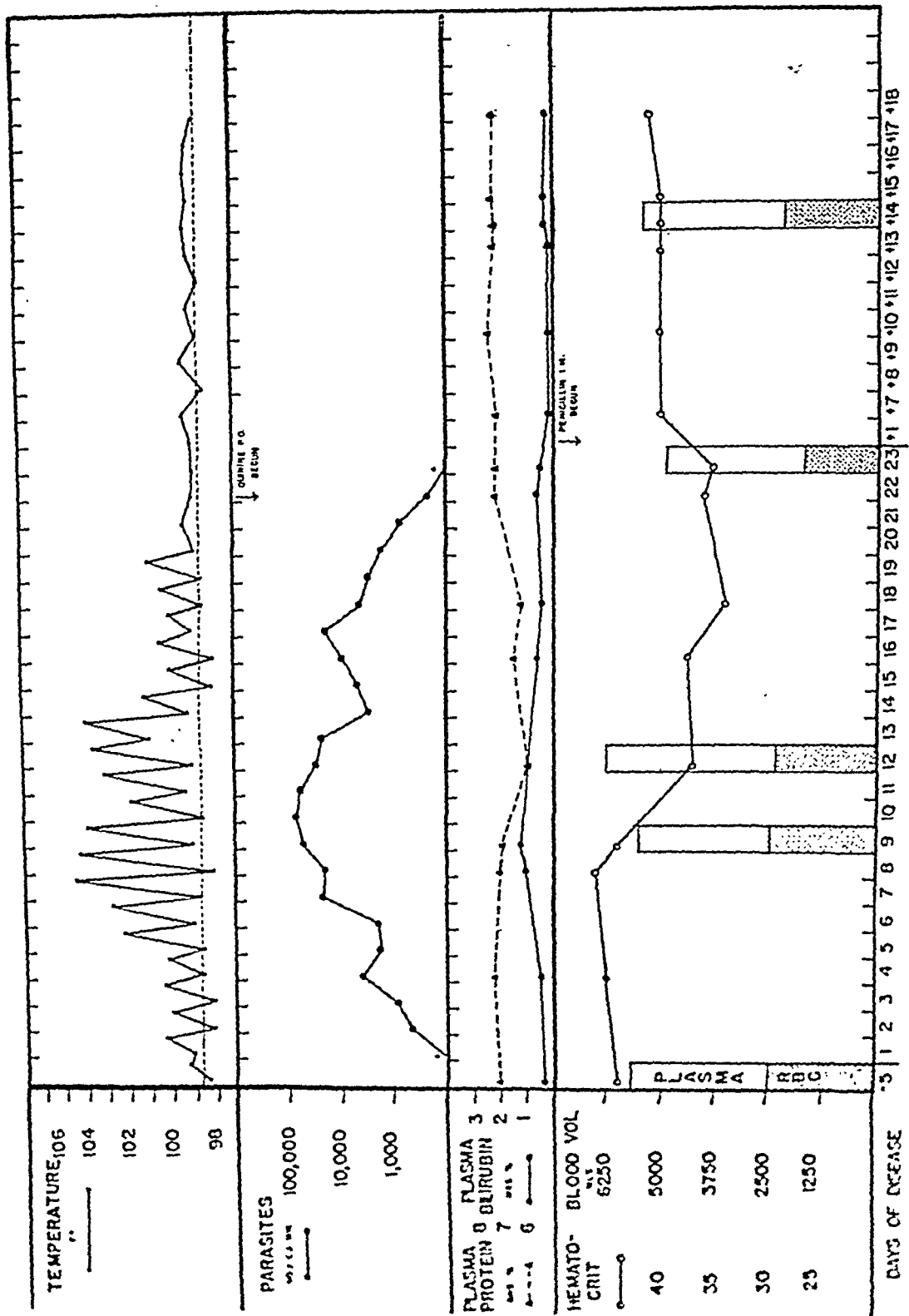


FIG. 2. COURSE OF PATIENT 8

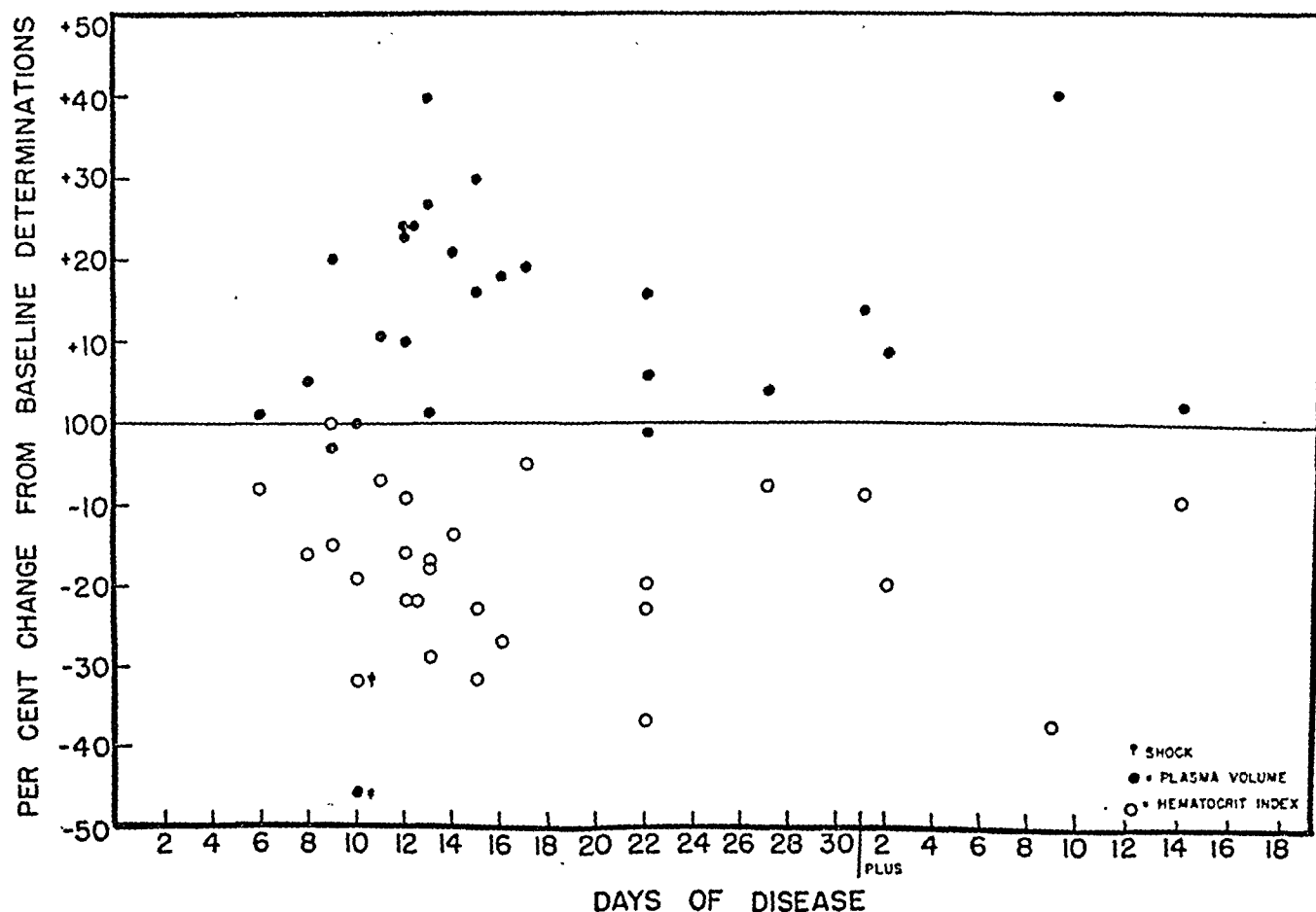


FIG. 3. RELATION OF PLASMA VOLUME TO HEMATOCRIT ALTERATIONS

DATA AND DISCUSSION

Plasma volume

Erythrocyte destruction is a constant concomitant of all active malarial infections. Hence, it was expected, *a priori*, that the plasma volumes of the patients studied would be increased if a stable blood volume were to be maintained. The data summarized in Table II tend to support this concept, for the plasma volume is increased almost without exception during the active phase of the *vivax* and *falciparum* infections studied. However, the plasma volume increase may exceed the calculated loss in circulating erythrocytes, resulting in hemodilution with a lowering of the venous hematocrit out of proportion to the actual decrease in total erythrocyte mass (Figures 1 to 3, and Table II, patients 5 and 8). This dilution phenomenon, which has also been observed in pneumococcal pneumonia (6), tends to occur shortly after the end of a paroxysm.⁶ The plasma volume tends to be lower during the paroxysm than dur-

ing the afebrile periods, probably the result of excessive loss of extracellular water by perspiration.

Erythrocyte mass

The mass of circulating erythrocytes was determined in all instances by subtracting the plasma volume from the calculated total blood volume. As would be anticipated in a disease in which there is continuing blood cell destruction, the erythrocyte mass progressively decreases with the course of the disease (Table II).

The validity of the value for erythrocyte mass obtained by the T-1824 method has been challenged by several workers (7, 8), who suggest that the calculated value is about 25 per cent higher than the actual value. However, since our experiments are concerned with alterations in total amounts rather than the total value *per se*, this error, if relatively constant, would appear to play a minor role in the interpretation of these data. The course of a patient who received 600 ml. of red blood cells immediately following a blood volume determination is illustrated in Figure 4.

⁶ The febrile excursion associated with schizogony, whether or not accompanied by a definite chill.

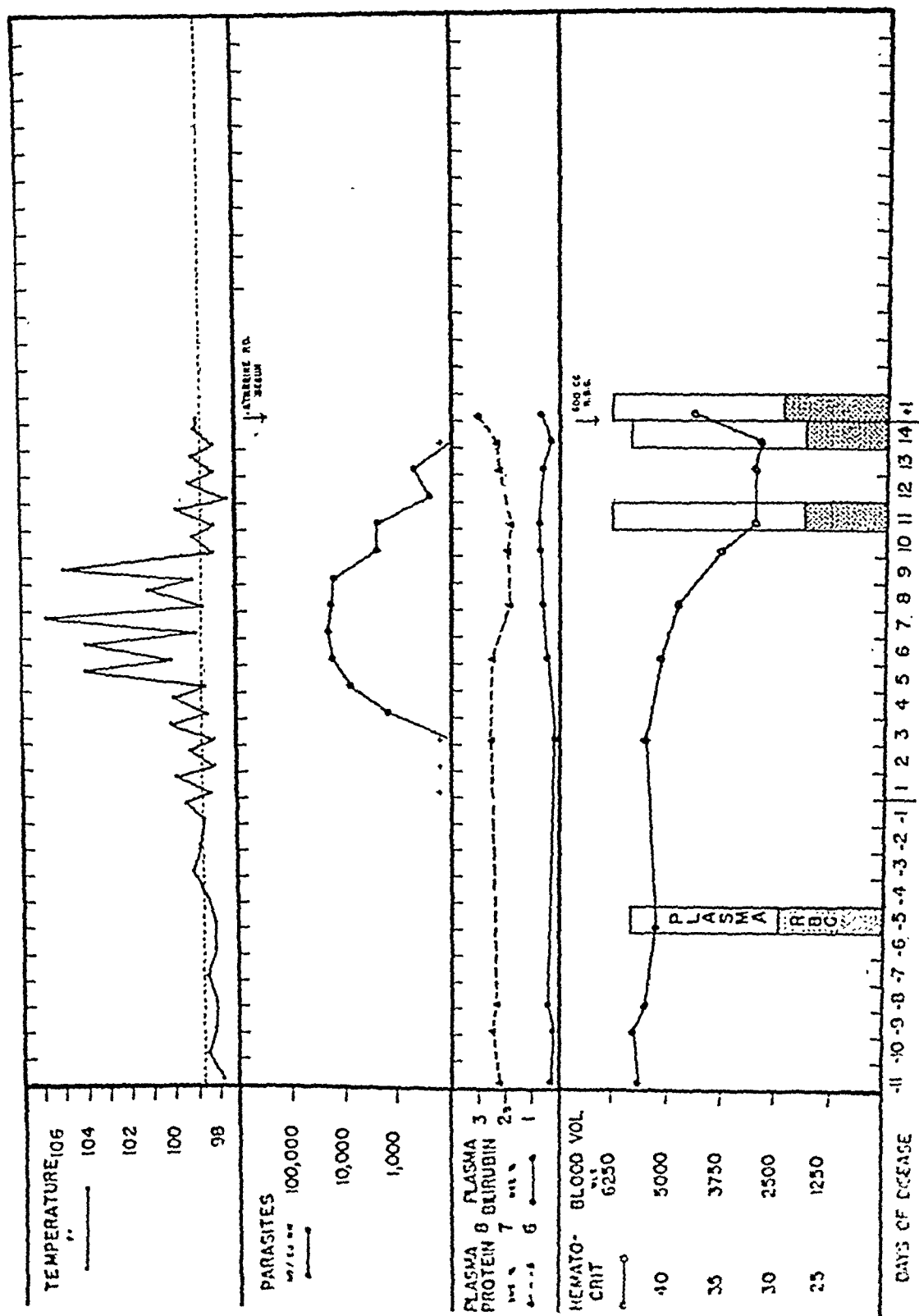


FIG. 4. COURSE OF PATIENT 4

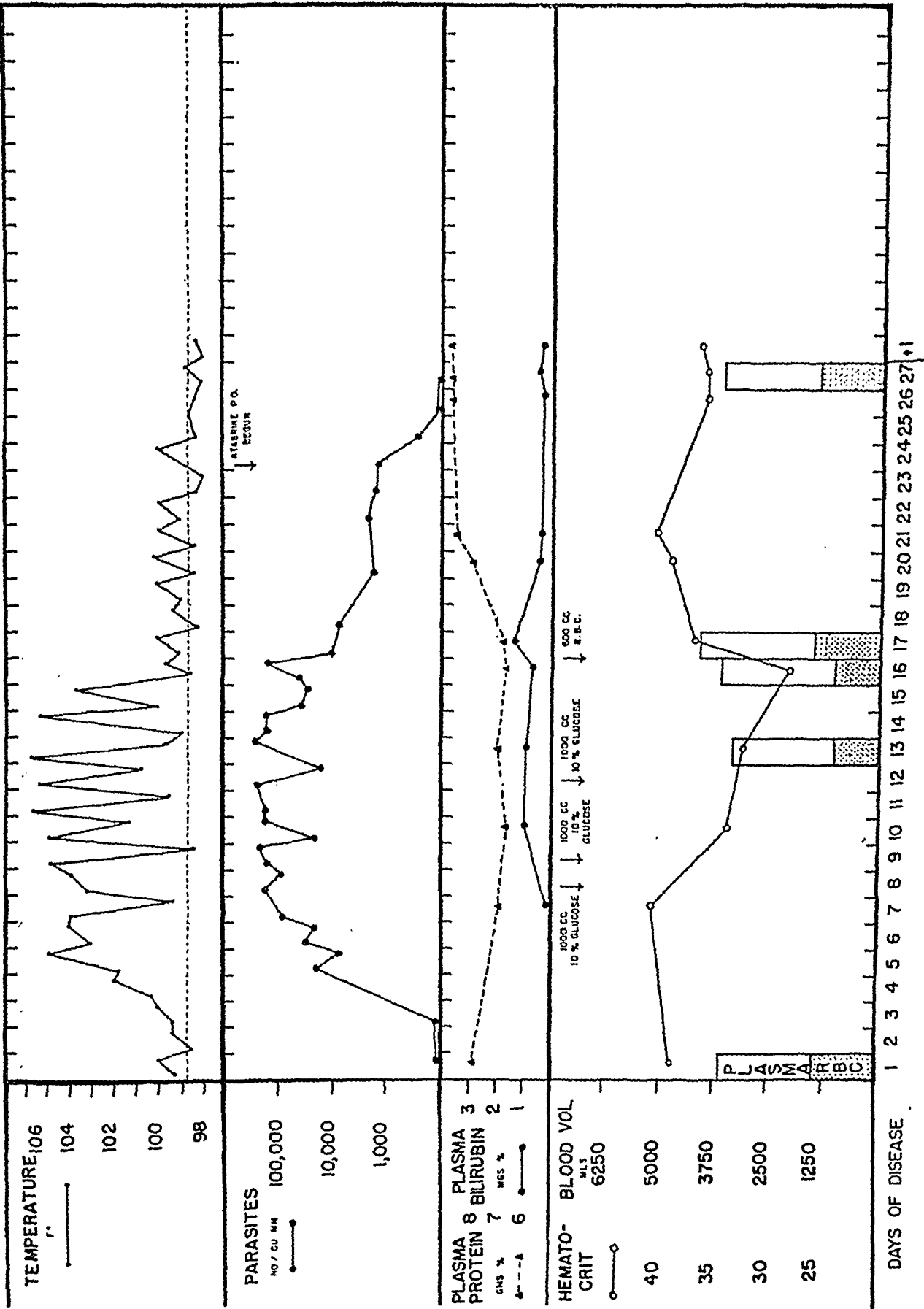


FIG. 5. COURSE OF PATIENT 7

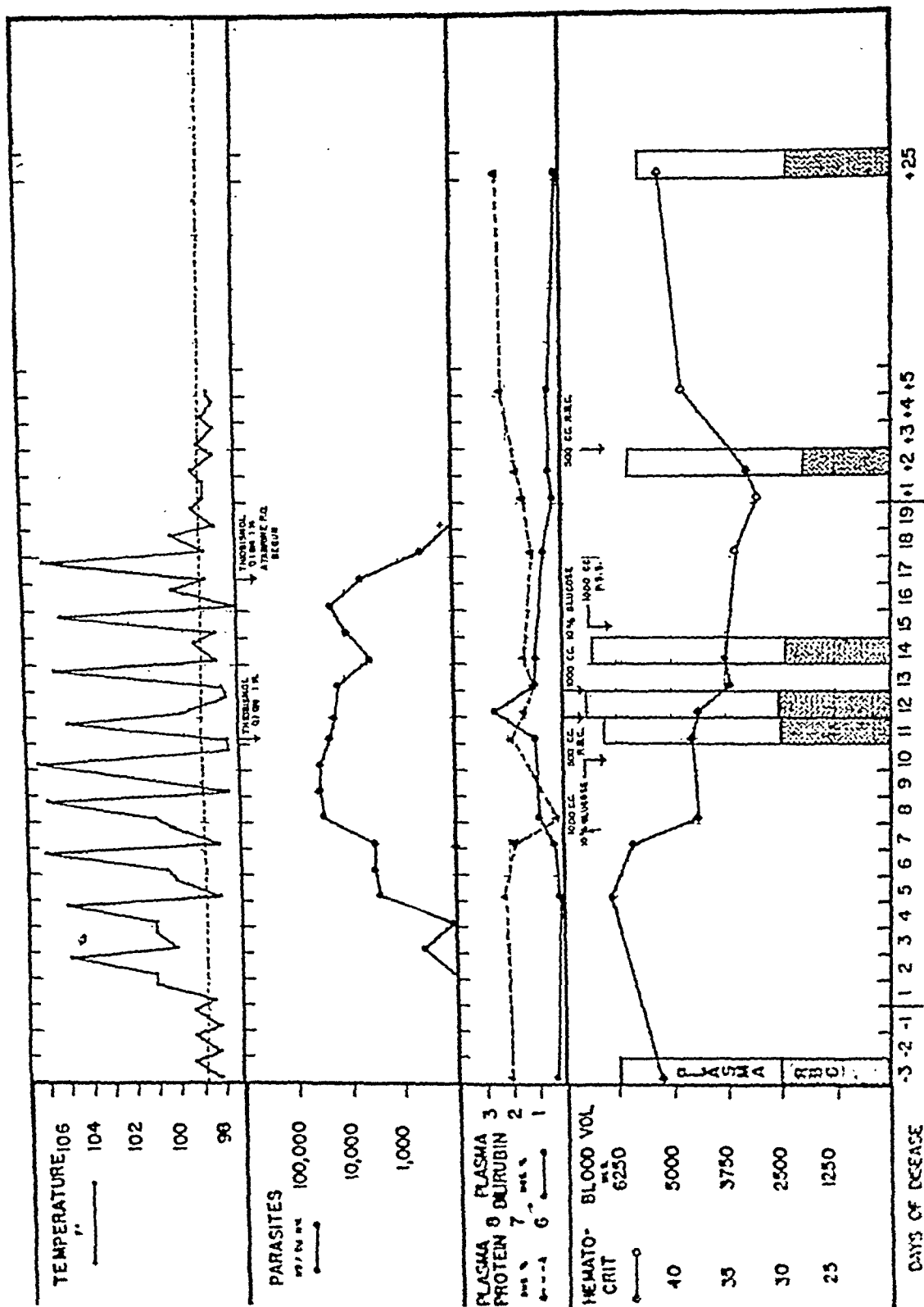


FIG. 6. COURSE OF PATIENT 1

On the following day another blood volume determination revealed that the red cell mass had increased by 530 ml. and the plasma volume by 50 ml. Another such patient, in whom 519 of 600 ml. of transfused erythrocytes were detected on the day following transfusion, is shown in Figure 5. Since the red blood cells were of necessity suspended in a small amount of plasma, the accuracy of the above determinations is probably greater than would appear. In 1 patient (Figure 6), however, the red cell mass increased only 98 ml. on the day following a transfusion of 500 ml. of erythrocytes, while the plasma volume increased 330 ml. This could be explained by the occurrence of a hemolytic transfusion reaction as reflected by the sharp increase in plasma bilirubin from 0.99 to 2.45 mgm. per 100 ml.

Total blood volume

The total blood volume, calculated from the hematocrit and the value for plasma volume, tends to be slightly lower during fever and higher during the immediate post-paroxysm, afebrile phase. These fluctuations, usually not great, result mainly from changes in the plasma component of the blood. Observations on 4 patients during various stages of convalescence (Table II, patients 1, 5, 6 and 8) suggest that the total blood volume tends to be decreased in convalescence primarily because the plasma volume does not remain sufficiently increased to make up for the reduction in red cell mass. The lowered plasma volume may result in part from the significant loss of body weight which these patients incur during the course of the disease.

Venous hematocrit index

A progressive fall in the venous hematocrit index begins on the third or fourth day after the appearance of parasites and continues for several days after their disappearance. This hematocrit fall reflects the direction of change but does not indicate the quantitative alterations in erythrocyte mass (Figure 7). The latter inaccuracy results, in many instances, from hemodilution, so that, for example (Figure 2), a drop of 7 points in the hematocrit on day 12 is due almost entirely to this phenomenon. In general, it may be said that in human malaria of the character reported in this

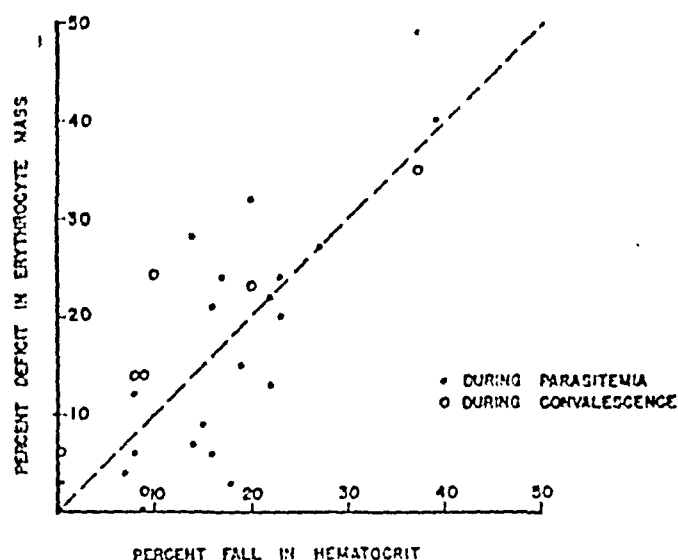


FIG. 7. CORRELATION BETWEEN DEFICIT IN ERYTHROCYTE MASS AND HEMATOCRIT FALL

study a single hematocrit index gives only an approximation of the true state of anemia, although a series of such determinations will point the direction of erythrocyte change.

Plasma bilirubin

The plasma bilirubin increases shortly after parasites appear in the blood and returns to normal following the end of the parasitemia. This increase, which is magnified considerably by calculation of the total circulating bilirubin (Table II), appears to be a reflection of the destruction of erythrocytes (Figure 8). As will be noted, this evidence of destruction may reach considerable proportions.

Anemia

These studies infer that at least 2 factors are operating in the production of the normochromic, normocytic anemia of malaria; i.e., erythrocyte destruction and hemodilution. Bone marrow studies (9) suggest the possibility of a third factor; namely, maturation arrest of the erythrocyte series.

Plasma proteins

The decrease in the concentration of plasma proteins during malarial infections has been reported (10, 11) to be due principally to the reduction of the albumin fraction. While our basic data are in agreement with the belief that the relative concentration of plasma proteins falls,

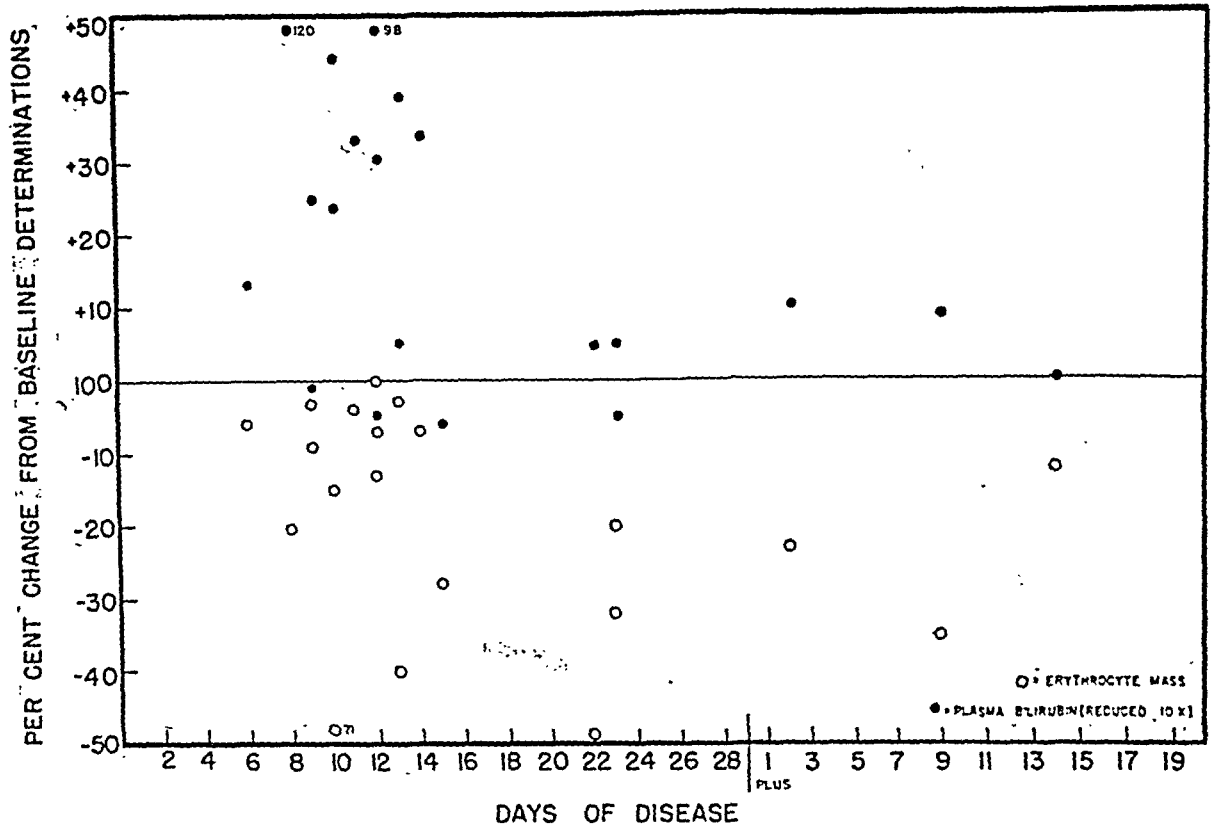


FIG. 8. RELATION OF PLASMA BILIRUBIN TO ALTERATION IN THE ERYTHROCYTE MASS

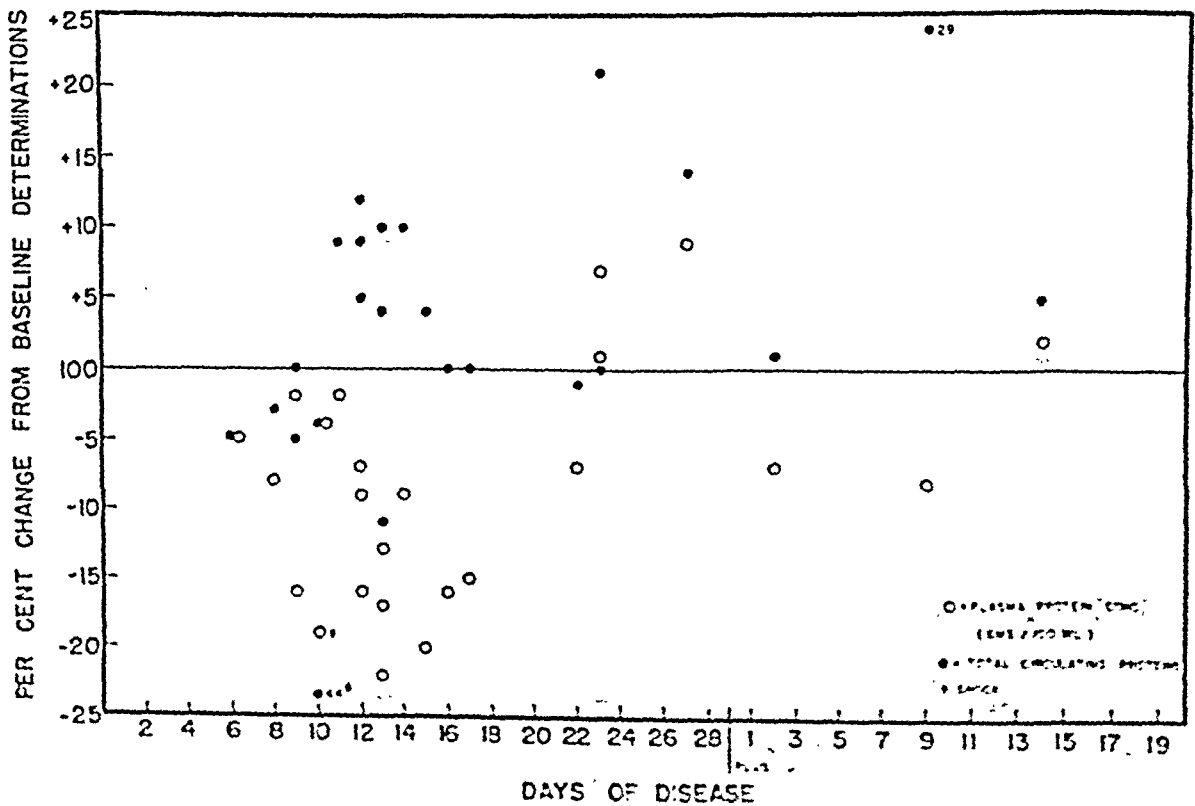


FIG. 9. RELATION OF CHANGES IN TOTAL CIRCULATING PROTEIN TO CHANGES IN PROTEIN CONCENTRATION (GRAMS PER 100 ML.)

the picture is altered when the total amount of circulating proteins is calculated (Figure 9), for it then becomes apparent that instead of a fall, there is more often no change or even a slight increase. While plasma dilution accounts for some of the fall in the concentration of plasma proteins during malaria, it fails to explain the reported (10, 11) selective plasma albumin decrease. A study now in progress, which combines blood volume studies with electrophoretic analysis of the plasma, may serve to clarify the question of quantitative alterations in the plasma protein fractions. Other factors which may play a rôle in the protein alterations, and which still remain to be appraised critically, are nitrogen imbalance, hepatic dysfunction, and the effect of the increased metabolic activity which accompanies fever.

Shock

During the course of these studies, 1 patient was encountered (Figure 10; Table II, Patient 2) who presented findings of sufficient interest to warrant a detailed discussion. This patient, on the tenth day of a severe *falciparum* infection, suddenly developed clinical signs of shock, including a rapid, almost imperceptible pulse, hypotension, moist, cooling skin, and semiconsciousness. Blood volume and other studies were performed while he was still in shock and prior to the administration of any intravenous fluids or medication. Instead of the anticipated elevation of the venous hematocrit, which is usually associated with the hemoconcentration of shock, the hematocrit index in this patient was 30, 32 per cent lower than the control value and 19 per cent less than the index obtained 2 days previously. The plasma protein concentration also fell. The plasma volume had fallen 46 per cent below the control value to 1628 ml., erythrocyte mass 71 per cent to 697 ml., and the total blood volume 57 per cent to 2325 ml. In other words, although there had been a drastic reduction in plasma volume, this was not indicated by the hematocrit because of marked and almost concomitant destruction of erythrocytes. The latter was reflected in the enormous increase in plasma bilirubin, which had first been noted 2 days before. It seems reasonable to assume that the anoxemia resulting from erythrocyte destruction initiated

the chain of events which terminated in the plasma decrease and shock. The marked reduction in total proteins, of the same general degree as the plasma volume, may be partly explained by increased capillary leakage.

The patient responded promptly to infusions of 500 ml. of erythrocytes and 500 ml. of plasma. Although he also received intramuscularly administered atabrine dihydrochloride, this could not have altered the immediate picture. Three days later the total blood volume had returned to normal with increases of 100 per cent above the shock levels in both plasma and erythrocyte volumes. The hematocrit, as expected, was still low. In spite of the slight increase in the concentration of plasma proteins, the calculated total amount of protein had increased greatly to a level slightly larger than the control value.

We have not been able to confirm these data on other patients as this is the only instance in which we have encountered this relatively rare complication of therapeutic malaria. The techniques employed were those used throughout this study; normal values were obtained on a control patient on the same day. Since the error which has been stated to occur in the study of shock by the use of T-1824 is thought to be due to increased loss of dye from the vascular bed and thus is responsible for plasma volume values which are inordinately high (12), the significance of the low values obtained in our patient would not be diminished by this factor. The correlation between the biochemical and volume studies done during this shock state, the studies done on prior and subsequent days, the clinical findings, and the theoretical concepts lead us to accept the qualitative accuracy of the direction and degree of the above described alterations.

The therapeutic implications of this case are clear, for had treatment been directed toward the improvement of shock by plasma transfusions alone, the clinical state undoubtedly would have been worsened. Although it was necessary to bolster the blood volume, to have done so without including erythrocytes might have been disastrous in view of this patient's marked anemia. Since the destruction of erythrocytes is such an integral part of malarial infections, it should be emphasized that transfusions of whole blood or erythrocytes are vital in the treatment of the shock which is not

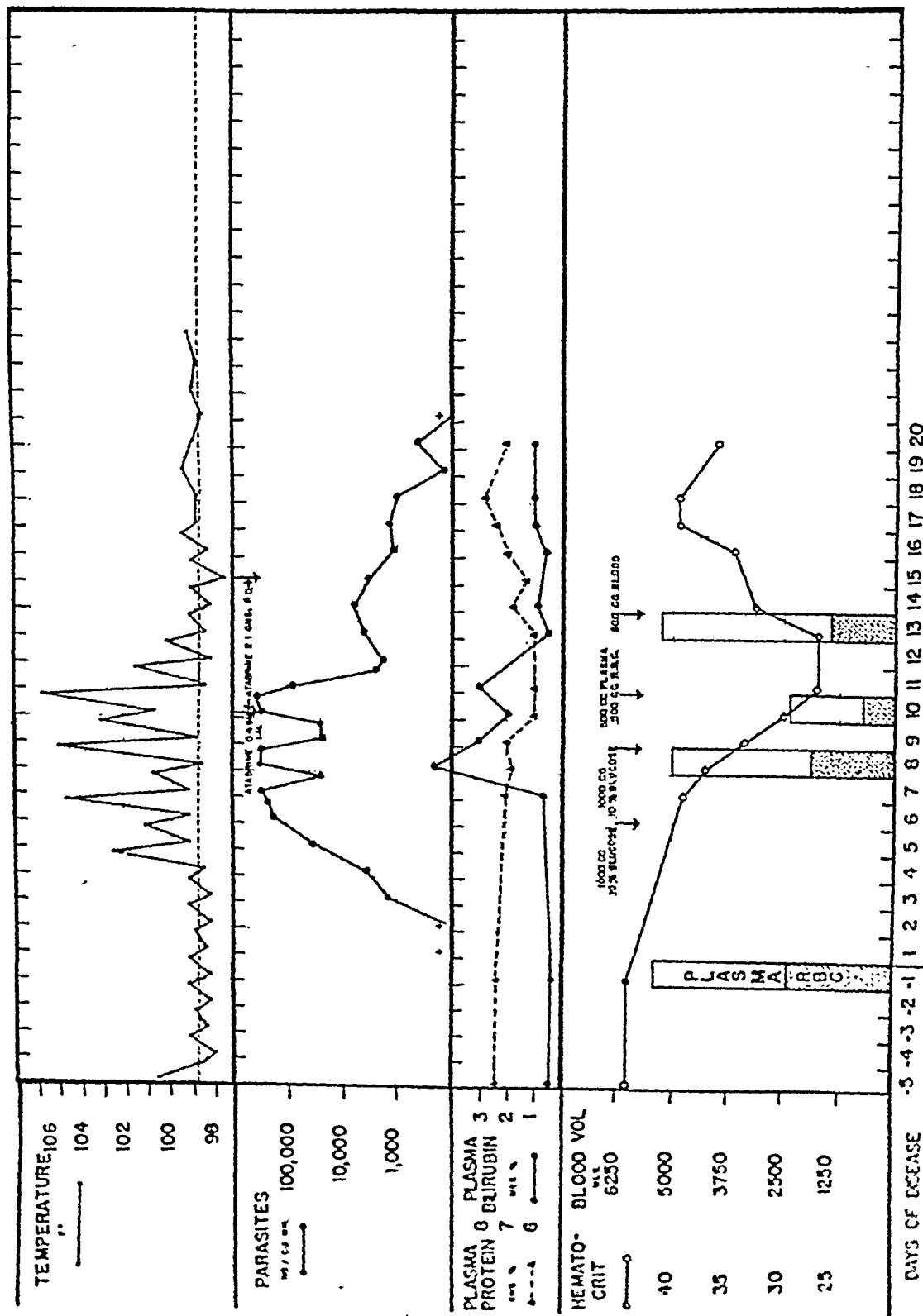


FIG. 10. COURSE OF PATIENT 2

uncommonly encountered in neglected *falciparum* infections. Plasma infusions alone may reduce the ratio of circulating red cells below the critical level and result in a fatality.

SUMMARY AND CONCLUSIONS

1. The alterations in blood volume during the course of induced malaria in 9 patients have been studied by means of the dye T-1824.

2. (a) Plasma volume is almost uniformly increased during the active phase of *vivax* and *falciparum* infections. This increase reflects the ability of the body to maintain a fairly constant blood volume in the presence of erythrocyte destruction.

(b) The plasma volume increase may be disproportionately great following a paroxysm and thus the degree of anemia as measured by the hematocrit may be exaggerated.

(c) The plasma volume tends to be lower during the paroxysm than during the afebrile intervals.

3. The calculated mass of circulating erythrocytes progressively decreases with the course of *vivax* and *falciparum* infections.

4. (a) The calculated total blood volume tends to be slightly lower during the malaria paroxysm and higher during the afebrile intervals. These fluctuations are due mainly to alterations in the plasma volumes.

(b) The total blood volume tends to be reduced during convalescence.

5. The concentration, as well as total amount of plasma bilirubin, increases during the period of visible parasitemia and reflects erythrocyte destruction.

6. The normocytic anemia of clinically active malaria may be due to at least two factors; namely, erythrocyte destruction and hemodilution.

7. The concentration of plasma proteins falls during the clinical phase of *vivax* and *falciparum* infections. However, the total amount of circulating plasma proteins remains fairly constant and may in fact be increased.

8. (a) In one case of shock due to severe *falciparum* malaria, a drastic decrease in plasma volume and erythrocyte mass was observed.

(b) These studies suggest that in the treatment of the shock which may be encountered in malarial infections, the administration of whole blood or erythrocytes is mandatory while plasma transfusions alone may be harmful.

It is a pleasure to acknowledge the constant advice and cooperation of Doctors Robert Briggs Watson, Henry Packer and William B. Wendel.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION

XXIX. SERUM ALBUMIN AS A DILUENT FOR Rh TYPING REAGENTS^{1,2}

By JAMES W. CAMERON AND LOUIS K. DIAMOND

(From the Departments of Physical Chemistry and Pediatrics, Harvard Medical School, and the Blood Grouping Laboratory, Children's Hospital, Boston)

During the last two years methods for the separation and concentration of the anti-Rh isoagglutinins have been developed as a part of the study of the fractionation of human plasma (1). The solubility characteristics on which the separation has been based resemble those of the anti-A and anti-B isoagglutinins, and have been summarized in a previous communication (2). The final products have been prepared as protein powders, stable and readily soluble, which may be packaged in convenient quantities and reconstituted to yield potent reagents useful for Rh typing or for A or B grouping, respectively.

In contrast to the anti-A and anti-B isoagglutinins, which may be obtained readily from grouped plasma pools from normal donors (3), the available supply of anti-Rh material is limited. The incidence of anti-Rh agglutinins in the general population is very low, and bleedings suitable as a source of original plasma for processing must be obtained from sensitized Rh negative donors, specially selected (2). Moreover, the phenomenon, termed "blocking,"³ whereby the antibodies in certain anti-Rh serums fail to agglutinate homologous Rh positive cells in sodium chloride solutions, but appear to combine with them and to inhibit the action of Rh agglutinins (4, 7, 8), has made these serums and the anti-Rh globulin

fractions obtained from them unsuitable for use as typing reagents by ordinary methods.

Anti-Rh agglutination with serums containing "blocking" antibodies may be demonstrated by the slide method⁴ if whole blood is used (9), or by the test tube method⁴ when compatible plasma or serum, instead of isotonic sodium chloride, is the reaction medium (10, 11). Plasma, or serum, is similarly effective with anti-Rh globulin fractions high in "blocking" antibodies (12). These globulin fractions, which in isotonic sodium chloride systems either on the slide or in the test tube, agglutinate specific Rh positive red cells poorly or not at all, cause strong agglutination in whole plasma. Plasma also intensifies, to a lesser degree, the reactions of "agglutinating" antisera, *i.e.*, those in which no "blocking" antibodies are demonstrable.

Influence of whole plasma on anti-Rh avidity

Reaction times of cells in plasma and in sodium chloride, when measured against each of these kinds of anti-serum, are reported in Table I. For these tests, packed Rh_i cells, twice washed in isotonic sodium chloride, were resuspended in their own plasma and in the salt solution. A measured volume, 0.03 ml. of cell suspension, was mixed on a slide with 0.01 ml. of antiserum, and the reaction was timed as the slide was rocked over a viewing box. The times for first agglutination visible to the unaided eye, and for an advanced stage, which has been called "complete" agglutination, were recorded. The maximum size of clump attained

¹ This work has been carried out under contracts, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

² This paper is Number 40 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry. The blood was collected by the Blood Grouping Laboratory, Children's Hospital, Boston.

³ The terminology of Wiener (4, 5) has been used throughout the paper. The terms "late" and "early" are alternatives to "blocking" and "agglutination," have been proposed elsewhere (6).

⁴ The slide method consists essentially of mixing, on an open glass slide, suitable proportions of cells and antibody solution, and then gently rocking the slide at 10 degrees the mixture for macroscopic agglutination. In the test tube method, cells and antibody are introduced successively into a small test tube, usually at 37° C. for about 1 hour. The tube is then centrifuged briefly, and the supernatant liquid is agglutination.

TABLE I

The avidities on the slide of an "agglutinating," and a "blocking" Rh antiserum, against cells in plasma and in isotonic sodium chloride

Type of antiserum	Rh ₁ cell concentration	Slide agglutination times				Size of final clumps*	
		First visible		Complete		In plasma	In sodium chloride
		In plasma	In sodium chloride	In plasma	In sodium chloride		
"Agglutinating"	per cent 50	15	20	60	100	lg	<lg
	25	15	25	70	210	lg	>sm
"Blocking"	50	20	25	110	>300	lg	sm
	25	20	40	120	>300	lg	<sm

* Symbols: "lg" means "large"; "sm" means "small."

within 5 minutes was also noted and was classified in a series from "small" to "large."⁵ It may be seen that against the "agglutinating" antiserum, a 50 per cent cell suspension in plasma reacted somewhat faster, and showed larger clumps than did the same cell concentration in sodium chloride. With a 25 per cent suspension the plasma medium gave a markedly better reaction than the sodium chloride medium. The contrast between plasma and sodium chloride was much more striking when "blocking" antiserum was used. Agglutination was excellent in plasma and very poor in the salt solution.⁶

Influence of plasma fractions on anti-Rh avidity

In an effort to discover which of the components of plasma contribute to the beneficial effects observed, a preliminary avidity study of some of the separated fractions of human plasma (1) was made. Approximately 10 per cent protein solutions were prepared at isotonicity and neutral pH. To detect any non-specific effects, 2 per cent suspensions of cells (type Rh₁) were first made in these solutions without the addition of antibody and were studied microscopically.

Fractions I (fibrinogen) and II (γ -globulin)

⁵ We have considered these slide reactions to be satisfactory if agglutination is first visible within 30 seconds and complete within 120 seconds and if the clumps are "medium" or "large."

⁶ These results are discussed further in connection with Table VIII.

caused non-specific clumping,^{7, 8} and therefore are not included in the study which follows. Subfractions of III and IV (described later) caused at most only a trace of clumping. No non-specific clumping occurred with fraction V (albumin) or with a similar solution of crystallized bovine albumin.⁹ Cell suspensions in whole plasma showed considerable *rouleau* formation, as is often the case.

The effects of the media upon speed of agglutination on the slide were next compared. Twenty-five per cent suspensions of Rh₁ cells were prepared in the various protein solutions, in whole plasma, and in sodium chloride alone. This concentration of cells, rather than a 50 per cent concentration, was chosen in order to observe more accurately the progress of cell aggregation. An anti-Rh globulin composed largely of "agglutinating" antibodies¹⁰ and dissolved in buffered sodium

⁷ Gelatin solutions in sodium chloride have a similar effect which is inhibited by glycine (13). Glycine likewise prevents the clumping of red cells by γ -globulin (14).

⁸ While the non-specific effects of both fractions decreased upon dilution with isotonic sodium chloride, an interesting difference in their influence upon avidity was noted. Fraction II, when used in 2 per cent concentrations as a cell suspension medium, caused no non-specific clumping, nor did it improve specific agglutination on the slide. Fraction I, on the other hand, showed no macroscopic non-specific effects at 0.8 per cent concentration, yet was still effective in improving avidity. Further studies are indicated.

⁹ See footnote number 12.

¹⁰ The antibody molecules themselves probably comprise less than 1 per cent of the protein in these preparations.

TABLE II
The effects of human plasma fractions on the avidity of anti-Rh globulin

Cell suspension medium *			Slide agglutination times	
Designation	Principal protein component	Final concentration on the slide	First visible	Complete
Isotonic sodium chloride	— — —	per cent	seconds	
Whole plasma	— — —	"5"†	110	>300
			40	120
Fraction III-0	β Lipoprotein	8	45	165
Fraction III-1, 2	γ Euglobulin	8	40	140
Fraction IV-1	β Globulin	8	40	210
Fraction IV-3, 4	α Lipoprotein	7	30	150
	α Globulin			
Fraction V (human)	Albumin	8	80	250
Fraction V (bovine)†	Albumin†	8	80	260

* 25 per cent suspensions of Rh₁ cells were used.

† "5 per cent" plasma means that 3 parts of cell suspension in whole plasma were added to 1 part of antibody reagent in sodium chloride.

‡ The material used here was a crystallized product, prepared from Fraction V by the Armour Laboratories, Chicago, Illinois. See also footnote 12 in text.

chloride was used; the tests were performed as described earlier in the paper. The results are presented in Table II.

Cells in sodium chloride, as expected, reacted very poorly, while those in plasma showed rapid agglutination. Several fractions of human plasma comprising lipoproteins and globulins, and known as Fractions III-0, III-1, 2, IV-1, and IV-3, 4, approached unfractionated plasma in their effects at the concentrations tested. The albumin fractions, both human and bovine, which had earlier been observed to increase speed of agglutination on the slide, were less effective than these fractions but much better than sodium chloride. Certain mixtures of albumin with the other proteins were more effective than albumin alone. Further study of the subfractions of III and IV is clearly indicated. Even though it was realized that these fractions might be useful, bovine serum albumin was chosen, because of its high stability, its low viscosity, and its availability in quantity, for additional experiments which are the subject of this paper.

Influence of serum albumin on anti-Rh agglutination by the test tube method

Several pools of anti-Rh plasma have recently been fractionated. The original material had been segregated into 2 categories, 1 containing mostly "agglutinating" antibodies, the other high in

"blocking" antibodies. The corresponding fractionation products have likewise been found to be low and high, respectively, in "blocking" antibodies.¹¹ The behavior of both kinds of material and the effects of albumin upon their agglutinating properties *in vitro* and upon their stability is considered in this communication. A related paper (6) deals with the use of albumin in the detection of anti-Rh agglutinins in whole serum. After tests had shown that human and bovine albumins are very similar in their effects, we adopted crystallized bovine albumin¹² for these experiments. A number of bovine albumin preparations, both of the crystallized protein and of Fraction V which is over 95 per cent albumin and the starting material for crystallization, have proved satisfactory.¹³ The reactions have always been noted in systems at neutral pH and made isotonic with sodium chloride or some other suitable electrolyte.

Albumin, like plasma, when used as part of the reaction medium, enables anti-Rh globulin high in

¹¹ The two varieties of antibody have not as yet been separated by chemical fractionation.

¹² We are indebted to the Armour Laboratories, Chicago, Illinois, for this material, which was prepared from a extract recommended by the Committee on Medical Research of the Office of Science Research and Development.

¹³ Certain very old preparations of crystallized bovine albumin have given equivalent results.

TABLE III

*Titration of anti-Rh globulin fractions high in "blocking" antibodies in sodium chloride systems with and without albumin**

Globulin preparation number	Final reaction medium	Type of cells †	Anti-Rh globulin dilution series ‡													
			1	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192
14	Isotonic sodium chloride	Rh ₁	0	0	0	0	0	0								
		rh	0													
	Albumin (20 per cent)	Rh ₁	++++	++++	++++	++++	++++	++++	+++	+++	+++	++	+	tr	0	0
		rh	0													
17	Isotonic sodium chloride	Rh ₁	tr	0	0	0	0									
		rh	0													
	Albumin (20 per cent)	Rh ₁	++++	++++	++++	++++	++++	++++	+++	+++	++	++	+	tr	tr	0
		rh	0													

* Geometric dilutions of the anti-Rh globulin solution were made in small test tubes so that each tube contained 0.05 ml. One-tenth of a ml. of a 2 per cent suspension of red cells was added to each tube. After incubation for 1 hour at 38° C., followed by brief centrifugation, the packed cells in each tube were gently shaken up and the degree of agglutination recorded. Weak reactions were checked microscopically. The symbol "tr" means "trace agglutination."

† "rh" means "Rh negative."

‡ Column headings in this and similar tables are reciprocals of the dilutions of the antibody reagent.

"blocking" antibodies, to agglutinate homologous Rh positive red cells. This is shown in Table III, which presents the titrations of 2 anti-Rh preparations assayed in sodium chloride systems with and without albumin. Preparation 14, which showed no titer in the salt solution alone, showed agglutination in albumin to a dilution of 1:1024. Preparation 17, with only a trace reaction at its highest concentration in sodium chloride, had a titer of 1:4096 in albumin. The reactions were specific, as was indicated by the Rh negative control tests.¹⁴

Further experiments have been performed with anti-Rh globulins containing "blocking" antibodies in sodium chloride systems with and without the addition of serum and albumin. Representative results are reported in Table IV. For each of these tests, 1 part of anti-Rh globulin solution in sodium chloride or albumin was mixed in a small test tube with 2 parts of a 2 per cent suspen-

sion of Rh₂ cells in sodium chloride, serum, or albumin. The final systems thus contained varying percentages of protein. No agglutination occurred with any of the anti-Rh preparations in the sodium chloride medium alone. Preparation 8 gave strong agglutination in all the other systems. Agglutination with Preparation 17 was weak in 5 per cent albumin, stronger in "5 per cent" serum, and still stronger in the higher protein concentrations. Agglutination with preparation

TABLE IV

*Agglutination by anti-Rh globulin fractions high in "blocking" antibodies, in sodium chloride systems with and without serum and albumin**

Final reaction medium	Globulin preparation number		
	8	17	2
0.9 per cent sodium chloride	0	0	0
5 per cent albumin	Solid†	+	0
"5 per cent" serum‡	++++	+++	0
10 per cent albumin	++++	++++	0
"5 per cent" serum and 5 per cent albumin	Solid	Solid	+++**
15 per cent albumin	Solid	++++	++

* The readings were made as in Table III, after the usual incubation and centrifugation. See text for further description.

† The term "solid" means "one large clump."

‡ Group AB, Rh positive. "5 per cent" serum means that whole serum was diluted 2:3 in the final system.

** Includes rouleaux.

¹⁴ The soluble A and B factors of Witebsky have usually been added to neutralize the anti-A and anti-B isoagglutinins. Complete elimination of agglutination due to these antibodies requires a large excess of the group-specific substances, particularly if the typing reagent is to be used in an albumin medium.

Non-specific reactions with Group 0, Rh negative cells have been observed in systems where relatively high concentrations of anti-Rh globulin had been superimposed upon 20 per cent albumin. Satisfactory typing reagents must of course be free from such effects. It is important, in the fractionation of anti-Rh serums, to remove as completely as possible γ -globulin and fibrinogen, which have been shown to be especially conducive to such reactions.

TABLE V

*Titrations of an anti-Rh globulin fraction low in "blocking" antibodies, in sodium chloride systems with and without albumin**

Type of cells	Final reaction medium	Anti-Rh globulin dilution series											
		1	2	4	8	16	32	64	128	256	512	1024	2048
Rh ₂	Isotonic sodium chloride	tr	+++	++++	++++	++++	++++	+++	++	+	tr	0	
	Albumin (20 per cent)	Solid	Solid	Solid	Solid	Solid	Solid	++++	++++	+++	+++	++	++
Rh ₁	Isotonic sodium chloride	++++	+++	+++	+++	+++	++++	++	++	+	tr	0	
	Albumin (20 per cent)	Solid	Solid	Solid	++++	++++	++++	++++	+++	+++	++	+	0
Rh'	Isotonic sodium chloride	++++	++++	++++	++++	+++	++	+	+	tr	0		
	Albumin (20 per cent)	Solid	++++	++++	++++	+++	+++	++	++	+	tr	0	

* The method used is described in a footnote to Table III. The term "solid" means "one large clump." The symbol "tr" means "trace agglutination."

2, which was very high in "blocking" antibodies by earlier tests, occurred only in the highest albumin concentration (15 per cent) and in the albumin-serum mixture.¹⁵ Albumin and serum enabled the antibodies to react to a degree dependent upon the nature and concentration of the proteins in these media.¹⁶ The total strength of antibody present and the relative amount of "blocking" antibodies no doubt also affected the final result with any particular preparation.

The reactions of an anti-Rh globulin very low in "blocking" antibodies will next be considered. Preparation 16 was made from serums containing for the most part "agglutinating" antibodies. Its titers against Rh₁, Rh₂, and Rh' cells in sodium chloride systems with and without albumin were measured, and in addition "blocking" tests were performed.¹⁷ The results are presented in Tables V and VI. It may be seen that agglutination with each of the 3 cell types was intensified in the albumin medium as compared with the sodium chloride medium. A definite prozone appeared in the lower dilutions of the series in sodium chloride against Rh₂ cells. This we have attributed to the presence of a small amount of "blocking" antibody, which decreased the agglutination in these dilutions. This explanation appears to be confirmed by the related "blocking test" against the

same Rh₂ cells in Table VI, since the standard anti-Rh₂ "agglutinating" serum was unable to produce its maximum effect at the lowest dilution of globulin. The series in sodium chloride against Rh₁ cells also showed a prozone, partly masked by agglutination due to anti-Rh' antibodies. This slight prozone was not reflected in the "blocking" test (Table VI). The Rh' series showed no "blocking" antibody by either test, yet its reactions were strengthened in albumin. Thus, when "blocking" antibodies were absent or present only in low concentration, albumin has also proven beneficial.¹⁸

TABLE VI

"Blocking tests" using the cells and anti-Rh globulin of Table V

Type of cells	Standard agglutinating serum	Anti-Rh globulin dilution series			
		1	2	4	8
Rh ₂	Anti-Rh ₂	+++*	++++	++++	++++
Rh ₁	Anti-Rh ₂	++++	++++	++++	++++
Rh'	Anti-Rh'	++++	++++	++++	++++

* Definitely weaker than succeeding dilutions.

No significant difference has been found in the titer endpoint of unfractionated, "agglutinating" serums whether or not albumin was present (6). The anti-Rh globulin in Table V likewise showed only a slight difference in this respect when

¹⁵ Rouleaux occurred in this mixture, but not in the

the titer in albumin was significantly higher than in sodium chloride alone.

Stability

High stability is important to the usefulness of blood typing and grouping reagents. Low moisture content (less than 1 per cent) and evacuation have been of aid in maintaining the potency both of anti-Rh and of anti-A and anti-B isoagglutinins. Nevertheless, additional means of assuring stability are desirable. Serum albumin has proved highly satisfactory for this purpose when used with anti-Rh globulin.

Experiments have been performed in which anti-Rh globulin samples were dried from the frozen state in isotonic sodium chloride with and without albumin. These samples were then heated as dry products at 50° C. for varying periods of time, after which they were redissolved and assayed together with unheated controls. Table VII presents the results of 1 such experiment in which an anti-Rh globulin low in "blocking" antibodies was used. In the titrations shown, the serial dilutions and the cell suspensions were in sodium chloride. Therefore, the only albumin present was that added before drying. The titration of an anti-Rh sample, dried with or without albumin and then heated, may be directly compared with its unheated control. The anti-Rh globulin, dried with albumin and heated for 8

days, showed little or no loss in titer against any of the 3 Rh positive cell types used. In contrast, titers of samples dried with sodium chloride alone, dropped so sharply after heating as to render the materials untrustworthy as typing reagents. Their avidities as observed on the slide were also greatly reduced, and the solutions themselves were more cloudy than before heating. The samples dried with albumin, on the other hand, showed no loss in avidity and yielded clear solutions. An anti-Rh globulin high in "blocking" antibodies has shown equal stability by appropriate tests, *i.e.*, its agglutinating properties as measured in an albumin system were maintained. A 42-day heat stability trial at 50° C. was made with an anti-Rh preparation dried with albumin as above. After this heating period, there was still no loss in titer or avidity against Rh₁ test cells. Other samples in albumin, with 0.1 gram per cent of carboxylmethoxylamine hemihydrochloride added as a preservative,¹⁰ were allowed to stand in solution at room temperature for periods as long as 30 days. At the end of this time, the samples showed some loss in

¹⁰ We are indebted to Dr. C. B. Favour of the Peter Bent Brigham Hospital for experiments which show that this antibiotic prevents the multiplication of common bacterial contaminants, unless these are present in very large numbers. These experiments were carried out under his contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

TABLE VII
*The stability at 50° C. of a dried anti-Rh globulin fraction, with and without albumin**

Diluent added before drying	Days heated dry at 50° C.	Type of cells	Dilution series of redissolved anti-Rh globulin									
			1	2	4	8	16	32	64	128	256	512
Isotonic sodium chloride	0	Rh ₁	++++	++++	++++	+++	+++	tr	0	0		
	8		+++	+++	++	tr	0	0				
Albumin (15 per cent)	0		Solid	Solid	Solid	Solid	++++	+++	++	+	tr	0
	8		Solid	Solid	Solid	++++	++++	+++	++	+	tr	0
Isotonic sodium chloride	0	Rh ₂	Solid	++++	++++	+++	++	+	tr	0	0	
	8		++	+++	++	++	tr	tr	0	0		
Albumin (15 per cent)	0		Solid	Solid	Solid	Solid	++++	+++	++	+	0	
	8		Solid	Solid	++++	++++	+++	+++	++	tr	0	
Isotonic sodium chloride	0	Rh'	+++	+++	+	tr	0	0				
	8		++	0	0	0						
Albumin (15 per cent)	0		Solid	++++	++++	++++	+++	++	tr	0		
	8		Solid	Solid	++++	+++	++	tr	0	0		

* The titration method is described in a footnote to Table III. The term "solid" means "one large clump"; the symbol "tr" means "trace agglutination."

both titer and avidity but were still useful typing reagents as measured with Rh₁ cells.

Variables affecting avidity on the slide

Among the factors which influence anti-Rh agglutination reactions *in vitro*, cell concentration, antibody concentration, and the nature of the reaction medium are of particular importance. In order to devise a slide typing test of maximum usefulness, the optimum relationships of these 3 variables were determined. The avidity studies with plasma fractions, discussed earlier in this paper, were concerned with the reaction medium. A further series of experiments has been performed to determine how avidity on the slide varies with varying cell, antibody, and albumin concentrations. Twice-washed Rh₂ cells and anti-Rh globulin were prepared separately in solutions of bovine albumin, isotonic with sodium chloride. A series of cell and globulin concentrations was used. An anti-Rh preparation high in "blocking" antibodies was chosen in order that any complications due to such material might be taken into account in arriving at a practical slide test method.

TABLE VIII

The effects of cell, antibody, and albumin concentrations on anti-Rh avidity on the slide

	Antibody solution		Rh ₂ cell suspension		Slide agglutination times		Size of final clumps*
	Anti-Rh globulin concentration	Albumin diluent concentration	Cell concentration	Albumin concentration	First visible	Complete	
Part I	<i>per cent</i>				<i>seconds</i>		
	6	20	50 25 10 2	20	13 18 40 >300	50 75 240 —	>lg <lg sm —
	3	20	50 25 10	20	13 24 60	55 130 >300	lg <md <sm
	1.5	20	50 25 10	20	20 40 90	110 180 >300	<lg >sm <sm
	1.5	20	50 in plasma		20	60	>lg
Part II	3	20 10 5 0	50	20 10 5 0	20 35 45 50	100 180 210 300	lg <lg >md >sm

* Symbols: "lg" = large; "md" = medium; "sm" = small.

The tests were performed as described earlier in this paper. The results are reported in Table VIII.

The speeds of agglutination and the sizes of clumps in Part I of this table may be directly compared with one another, since the tests were all performed within a period of a few hours. The times shown in Part II are relatively slower, having been observed with cells 24 hours old, but are likewise comparable among themselves. It may be seen that lowering the concentration of any one of the 3 variables decreased both speed of agglutination and size of clumps. The system was particularly sensitive to cell concentration; that is, 25 per cent cell suspensions gave much poorer results than 50 per cent, and 10 per cent suspensions were extremely poor, even though a high concentration of albumin was present. Reducing the concentration of the anti-Rh globulin was less critical. A system with 50 per cent cells and 20 per cent albumin showed very good agglutination (20", 110", <lg) even when the anti-Rh globulin was as low as 1.5 per cent, which is only one-fourth of the maximum concentration tested.²⁰ For a comparison of reaction mediums, a 50 per cent cell suspension in whole plasma was tested. The reaction of this suspension was faster and showed larger clumps than the comparable 50 per cent suspension in albumin when the same antibody concentration was used with both. The effects of reducing the albumin concentration, using a constant 50 per cent cell suspension, are shown in Part II of Table VIII. Each reduction resulted in a poorer reaction upon the slide.

The high sensitivity of "blocking" antibodies to cell concentration on the slide is in agreement with other findings (9). In those experiments, however, diluted cell suspensions were made by adding isotonic sodium chloride to a plasma medium. In Table I of the present paper comparisons of slide agglutination with 50 per cent and 25 per cent cell concentrations, each in an undiluted plasma medium, were presented. In this medium, against both "blocking" and "agglutinating" anti-serums, there was no significant difference in the behavior of the 2 cell concentrations.

It appears, therefore, that under proper conditions, any one of the 3 variables here discussed

²⁰ The cell and anti-Rh globulin concentrations referred to are those before the reagents were combined on the slide.

may be a limiting factor, particularly in the presence of "blocking" antibodies. With whole plasma as the reaction medium, high cell concentration is not as essential as it is with an albumin medium. On the other hand, high antibody concentration can at least partly offset the effects of lower cell or albumin levels. By specifying a 40 to 50 per cent cell suspension in plasma or in 20 per cent albumin, definite economy may be achieved as regards the concentration of anti-Rh globulin necessary to provide a fast, accurate reaction on the slide.

DISCUSSION

The usefulness of many anti-Rh serums as typing reagents has been sharply limited by interfering phenomena which have been attributed to "blocking" antibodies. Agglutination often fails to occur with such materials when the usual isotonic sodium chloride medium is used for testing. The fractionated and concentrated anti-Rh typing globulins often behave in a similar manner when they contain "blocking" antibodies.

Cell agglutination takes place *in vitro*, however, in the presence of "blocking" antibodies if the medium of reaction is plasma. Plasma is particularly useful as a suspension medium when the unknown cells are to be typed by the slide method. Since high cell concentrations are desirable, the samples may be taken and used as whole blood; or cells from clotted blood samples may be recovered, in concentrated suspension, in their own serum. As a routine diluent for Rh typing by the test tube method, which requires dilute cell suspensions and may include titrations, plasma is less satisfactory. Except when obtained from bloods of group AB, any stock plasma to be used with a variety of cell samples must be completely neutralized for the anti-A and anti-B isoagglutinins. Plasma is not highly stable when stored as a liquid, and often causes *rouleau* formation which confuses microscopic readings.

The value of serum albumin in making "blocking" antibody products useful as typing reagents, and in stabilizing anti-Rh globulin fractions, has been demonstrated by the experiments reported. Much plasma, or serum, formerly considered useless because of the presence of "blocking" antibodies, may now be fractionated and the products used in conjunction with albumin. Since a high

concentration of albumin is required with such preparations, it is useful to supply this material both as a constituent of the dried anti-Rh reagent and as an auxiliary diluent. Typing tests by the slide and the test tube methods can then usually be performed with the same anti-Rh globulin solution.

The properties which enable plasma and its components to bring about agglutination in the presence of "blocking" antibodies are of interest.²¹ Since a much higher concentration of serum albumin than of whole plasma is necessary to obtain comparable results, it must be concluded that factors other than albumin are effective in whole plasma. Several plasma fractions improved the avidity of anti-Rh globulin to a greater extent than did albumin, even when the antibodies present were largely of the "agglutinating" variety. Other preliminary experiments have indicated that these fractions are also effective as reaction media with "blocking" antibodies. Since the principal protein components of the various fractions differ widely from one another, no single specific protein would seem to be required.

The relationship between specific and non-specific effects of proteins upon red cell agglutination may be significant. Plasma fractions which caused non-specific clumping at high concentrations were usually among those which most improved specific agglutination at lower concentrations. An investigation of the effects of "blocking" antibodies and of various proteins on the electrical potentials of Rh positive and Rh negative red cells would be of interest. It has been reported (15) that changes in potential accompany isoagglutination.

Note added in proof: In a paper just published (Nature, 1945, 156, p. 233) Coombs and Race have shown that changes in the mobility of red cells in an electric field occur after treatment with either "blocking" or "agglutinating" antibodies.

Fractionation has not been found to alter the properties of "blocking" antibodies. Plasma pools high in these substances yield anti-Rh globulin fractions of the same nature. This may be interpreted as evidence that the "blocking" antibodies are real entities and not merely the effects

²¹ An explanation for the effect has recently been suggested (10).

of the original plasma environment. Since, under certain conditions, strong agglutination occurs in the presence of these antibodies, it is important to determine whether this reaction involves the "blocking" antibodies or is caused by "agglutinating" antibodies, present in the mixture but not always manifest.

SUMMARY

1. The presence of "blocking" antibodies heretofore has seriously limited the usefulness of many anti-Rh serums and globulin fractions as typing reagents for the detection of the Rh factor in red cells.

2. Plasma as a reaction medium is known to enable agglutination to occur *in vitro* with these materials.

3. Several different fractions of plasma have been found to improve the avidity of anti-Rh globulin preparations.

4. Serum albumin in high concentrations enables agglutination to occur with anti-Rh globulin fractions high in "blocking" antibodies and stabilizes anti-Rh globulin in general. Albumin as a reaction medium possesses certain technical advantages over plasma.

5. The influence of erythrocyte, antibody, and albumin concentrations on anti-Rh avidity on the slide has been studied.

6. Practical applications of the experiments are discussed, and the significance of certain observations is considered.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION

XXX. THE USE OF SALT-POOR CONCENTRATED HUMAN SERUM ALBUMIN SOLUTION IN THE TREATMENT OF CHRONIC BRIGHT'S DISEASE^{1, 2, 3}

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Much of the disability induced by chronic Bright's disease, particularly during the nephrotic stage, is attributable to the coexistent disturbances in protein metabolism. The latter may be characterized as follows: 1, proteinuria; 2, hypoalbuminemia; and 3, depletion of tissue protein stores. During the past few years, numerous attempts have been made to correct the hypoalbuminemia, depletion of body protein stores, and attendant edema in patients with nephrotic syndromes by administering human plasma intravenously (1, 2, 3). This form of therapy obviously had as its objectives the use of protein solution as a diuretic agent by virtue of its high colloid osmotic pressure, the restoration of serum albumin level, and the replenishment of body protein stores. The last two objectives were rarely, if ever, attained because of the limited quantity and high cost of human

plasma solutions. The former objective was frequently not accomplished, even with concentrated solutions of human plasma, possibly, as has been suggested (1), because of the relatively high content of sodium chloride in these preparations.

Recently it has been shown (4) that a single dose of 25 grams of concentrated high-salt human serum albumin proved inadequate to induce any prolonged changes in serum protein level or clinical condition although small but consistent increases in urine volume were observed in all three nephrotic patients following therapy. In another study (5) the administration of considerably larger amounts over longer periods led to inconstant results. As in the case of plasma, the salt content of the albumin used in these studies was relatively high, as considerations of stability under field conditions for which the concentrated normal human serum albumin was designed led to the adoption of a concentration of 0.3 M. sodium chloride (6).

Recently there has been developed a series of preparations of normal human serum albumin whose stability characteristics are even better than those of the high-salt albumin, but whose salt content is extremely low (7). In the following table is presented a comparison of the amount of sodium (expressed as sodium chloride) contained in the protein dosage sufficient to hold 1 liter of plasma water in the blood stream in the normal range of plasma colloid osmotic pressure.

Citrated plasma	High-salt albumin	Salt-poor albumin
11.7 gm.	4.8 gm.	1.6 gm.

Though limitation of these preparations has made direct comparison impossible in the studies reported here, we have attempted to answer the following questions:

¹ The albumin used in this work has been prepared under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

² This is paper No. 41 in the series, "Studies on Plasma Proteins" from Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

³ This article has been released for publication by the Division of Publications of the Bureau of Medicine and Surgery of the United States Navy. The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting views of the Navy Department or the Naval Service at large.

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1. Is salt-poor concentrated human albumin a safe and effective diuretic agent in edema of renal origin?

2. What is the optimum dose and method of administration?

3. What effect does the administration of salt-poor concentrated human albumin have on the serum protein level of patients with chronic nephritis?

4. What effect does the intravenous administration of a concentrated albumin solution have on nitrogen balance in the presence of adequate dietary protein?

METHODS

The salt-poor albumin used in these investigations was prepared in the form of a 25 per cent solution without preservative by the Plasma Fractionation Laboratory of the Department of Physical Chemistry.⁷ The physical chemical characterization of normal human albumin has been presented in previous papers of this series (8 to 11). The albumin was diluted with sterile dextrose solution so that the resulting fluid contained 10 per cent albumin and 6 per cent dextrose.⁸

During these studies, patients were restricted to bed rest and were maintained on a constant regimen.⁹ Each patient received a diet of constant composition containing at least 1 gram of protein per kilogram of body weight per day and adequate in carbohydrate and fat to meet caloric requirements. The diet was salt-poor in all cases except that of K. N. who received a "restricted salt" diet.¹⁰ The daily fluid intake of each patient was also maintained at a constant level. On those days when albumin solution was administered, an equivalent volume of fluid was subtracted from the patient's drinking water.¹¹

⁷ We are indebted to Dr. L. E. Strong and the Staff of the Plasma Fractionation Laboratory for the carrying out of the preparation, and to Dr. Geoffrey Edsall and the Staff of the Antitoxin and Vaccine Laboratory of the Massachusetts Department of Public Health for control of sterility and safety.

⁸ Pyrogen reactions were observed rarely; in one instance this was traced to the albumin itself, in the other to the dextrose solution used in diluting.

⁹ We are indebted to Dr. Thomas J. Kennedy for his aid in the supervision of this regimen and of many of the experiments.

¹⁰ The salt-poor diet on the Metabolism Service of the Peter Bent Brigham Hospital contains 1 to 2 grams of salt per day; the "restricted salt" diet contains 5 to 6 grams of salt per day.

¹¹ In the initial studies the pulse, respiration, blood pressure, and venous pressure of the patients showed little variation; these observations were later discontinued. In our case only, that of R. S., did the intravenous administration of albumin cause a significant rise in blood pressure.

All blood samples were drawn in the morning while the patients were fasting. The urine of each patient was collected quantitatively, pooled in 12- or 24-hourly amounts, and preserved with toluol and refrigeration. Stool specimens were collected for 3- or 5-day periods and were dried at once on a steam-bath. In calculating nitrogen balance, a value of 15 per cent of nitrogen intake was used for the value of nitrogen excretion in the stool in all instances since stool analyses on several occasions indicated that the fecal nitrogen excretion in these patients did not exceed this value.

Blood sera were analyzed for total nitrogen by micro-Kjeldahl digestion followed by direct Nesslerization. Nonprotein nitrogen of blood and urine was determined by Nesslerization following micro-Kjeldahl digestion of a trichloroacetic acid filtrate.¹² Serum albumin and globulin were determined chemically by the method of Howe (13) using the arbitrary nitrogen factor 6.25, urea nitrogen by a modification of the method of Karr (14), and cholesterol was estimated colorimetrically by direct application of the Liebermann-Burchard (15) reaction on an alcohol-ether extract without hydrolysis of esters.

In electrophoretic analyses¹³ carried out in the apparatus of Tiselius (16), the long cell described by Longsworth (17) was used. Schlieren diagrams were obtained by the cylindrical lens method (18). Sodium diethyl barbiturate buffer at pH 8.6 and ionic strength 0.1 permitted, in most cases, adequate separation of albumin from alpha-1 globulin when the run was carried out at a field strength of approximately 5 volts per centimeter for 2 or 3 hours at 2° to 4° C.

Because of the abnormally large amount of lipoids bound to the non-dialyzable protein nitrogen and migrating principally with the beta (19, 20) and alpha globulins, the application of electrophoretic distribution of compo-

¹² Beckman and associates (12) have reported considerable amounts of a trichloroacetic acid soluble protein in the urine of patients exhibiting proteinuria of etiology unspecified. In electrophoretic mobility at pH 8.6 this protein appeared intermediate between alpha-1 and alpha-2 globulins. In that both blood and urine of patients with chronic Bright's disease contain abnormally high amounts of protein of similar electrophoretic characteristics, it is possible that the use of trichloroacetic acid as a precipitant introduces a systematic error in nonprotein nitrogen and, thus, by difference, in protein nitrogen determinations.

The fact that the error is small in the instance of serum protein values is indicated by N.P.N. values within normal range in these experiments. Whereas in the case of urine the error in partition between protein and nonprotein nitrogen may be larger, it will not affect either the overall nitrogen balance studies nor, indeed, the data obtained on changes in protein excretion following albumin administration in that albumin is insoluble in trichloroacetic acid and the administration of albumin appeared not to affect significantly the daily output either of nitrogen or nitrogen of electrophoretically determined protein.

¹³ We are indebted to Mr. Merrill J. E. Hoffa for carrying out most of these analyses.

nents to total protein nitrogen figures in order to determine the plasma concentration of a given component is frequently subject to extraordinary error.¹⁴ Likewise, difficulty in extracting dye T-1825 from highly lipemic sera rendered grossly inaccurate several attempts at blood volume determinations.¹⁵ Therefore, in the studies here reported, no attempt has been made to calculate from electrophoretic schlieren distribution, protein nitrogen, and

¹⁴ This error, far greater than that encountered in normal plasma (21), applies to a much less degree in the instance of nephrotic urine owing to the rather small amount of lipid bearing protein which passes through the kidney. In the following table are presented the refractive index increments per gram of nondialyzable plasma, and urinary protein nitrogen of patient J. G., during a control period and following the injection of albumin. The plasma

	Refractive index increment <i>per gram protein N</i>	Uncorrected albumin content <i>per cent</i>	Corrected albumin content * <i>per cent</i>
Plasma			
Control period	2.07×10^{-4}	9	17
Following intra- venous albumin	1.63×10^{-4}	32	45
Urine			
Control period	1.20×10^{-4}	64	66
Following intra- venous albumin	1.17×10^{-4}	88	89

* The refractive index increment per gram of normal human serum albumin nitrogen has been found in this laboratory to be approximately 1.16×10^{-4} . The refractive index correction is based on this value although the actual value of nephrotic serum albumin may be somewhat higher owing to migration of some lipid with it. The correction for the effect of protein concentration and ionic strength on apparent albumin concentration, which is less in amount and opposite in sign, has been neglected in this table.

albumin content corrected on the basis of the refractive index increment differs markedly from the uncorrected value obtained directly from the schlieren diagram, the urinary albumin but slightly. The magnitude of correction will vary with the degree of lipemia. The careful addition experiments of Luetscher (4) demonstrated that in his studies such errors were negligible.

¹⁵ We are indebted to Dr. John G. Gibson, 2nd, for both carrying out and providing the following critical comment on the determination of the plasma volume of certain of the patients by the Evans blue technique.

"The turbidity of these patients' sera is such as to cause a great deal of deflection of incident light in the absorption cup. Even when each individual dyed plasma sample is read at a wave length of 620 against dye-free plasma, the results obtained do not reflect the light absorption due to the presence of dye alone. Extraction with tri-ethyl-phosphate, which is ordinarily adequate in dealing with moderate lipemia, failed in the cases of these milky plasmas. No method of dye extraction is to my knowledge available which will overcome this difficulty."

The practice of Longsworth and co-workers (19) of brief centrifugation at 30,000 RPM was not attempted.

plasma volume the total circulating amount of any given component. The electrophoretic distributions have in the main been used as a check on the direction of change of serum albumin as shown by the Howe (13) method and to indicate any gross shifts in individual globulin components as a result of therapy. Although approximations in change of plasma volume have occasionally been calculated from hematocrit changes, the limitations of this method are clearly recognized, particularly the error introduced by change in red cell volume during albumin infusion.

With the variations in the clinical course and physiologic factors known to occur in chronic Bright's disease, it is apparent that a therapeutic agent which might be helpful at one stage of the disease might be of no help at another time or might even be contraindicated. Thus, it is essential to study the same patients in various stages of the disease throughout their long course of illness, or else one must include in the group of cases under observation representatives of the several stages of the disease. Fully aware of the changes in the clinical course and inconstancy of effectiveness of therapeutic agents, we have studied a small group of patients with chronic nephritis in which certain of the classical stages are represented. Detailed clinical summaries are recorded at the end of the paper.

Distributions of components in the electrophoretic schlieren diagrams of the plasmas of these untreated patients are presented in Table I and illustrated together with certain clinical data in Figure 1. It is of interest to correlate the range of these distributions with the clinical stages. At one extreme is patient J. G., whose massive edema, severe hypoproteinemia, albuminuria, and extreme hypercholesterinemia constituted the nephrotic picture in its most severe form. The schlieren diagram at the top of Figure 1 likewise shows striking deviations from the normal that have been often noted in nephrosis (4, 19, 20). Albumin constitutes less than 10 per cent of the diagram in contrast to a normal value of 55 per cent. Likewise, the gamma globulins comprise less than half their normal area. The principal components are alpha-2 globulins and beta globulins, of which alpha-2 are present in excess of beta. The fibrinogen is elevated.

Patients L. I., W. H., D. S., and K. N., who clinically present the nephrotic picture in progressively less marked degree, show in the elec-

TABLE I

Distributions of components in electrophoretic schlieren diagrams of plasma proteins of patients before albumin administration*

Patient	Albumins	α_1 Globulins	α_2 Globulins	β Globulins	Fibrinogen	γ Globulins
	per cent					
J. G.	7	4	42	28	16	3
L. I.	17	5	36	22	16	4
W. H.	17	5	21	38	15	4
D. S.	26	8	22	30	9	5
K. N.	37	6	15	22	12	8
E. B.	46	4	34**		13	3
R. S.	32	5		23	(clotted)	20
Normal pooled human plasma	55	5	9	13	7	11

* Sodium diethylbarbiturate buffer, pH 8.6.

** Not resolved.

trophoretic diagrams a progressively greater contribution of albumin to the total area and a trend toward the restoration of the normal beta-alpha-2 ratio wherein the beta peak is predominant.

At the other extreme of the group is patient R. S. He alone presents a gamma globulin which, in contrast to the subnormal value in the other patients, comprises almost twice the normal contribution of this component to the total picture. In this respect, the electrophoretic diagram resembles that of acute rheumatic fever (22, 23), of disseminated lupus, (23, 24) and of periarteritis nodosa (23).

In view of the fact that in these latter diseases an immunological component in the mechanism has long been suspected, that in certain forms of experimental nephritis such a component has been demonstrated (25), and also in view of the fact that the gamma globulin fraction in normal human plasma contains the majority of the antibodies (26), it is of particular interest that this patient alone gave evidence of recent acute exacerbation of his renal disease in the form of considerable oliguria and hematuria.¹⁶

In Figure 2 are presented electrophoretic schlieren diagrams with the plasma of patient J. G. before and after a remission in her disease. It will be noted that her plasma protein components appear to progress through the same changes as are illustrated in passing from severe to mild ne-

phrosis in the group of patients detailed in Table I and Figure 1.

OBSERVATIONS

1. Dosage and method of administration

In the treatment of shock in war casualties, for which concentrated serum albumin was originally developed, rapid injection was desirable to effect an immediate increase in plasma volume. In patients with chronic nephritis and edema, however, it was obvious that a slower rate of injection might be required if circulatory failure were to be avoided. In exploratory experiments the intravenous administration of 10 grams of salt-poor albumin per hour occasioned small rises in pulse rate, blood pressure, and venous pressure. This rate appeared well within limits of safety for initial injection in all patients studied. Faster administration proved uneconomical owing to increased urinary protein loss in patients with marked proteinuria.

In order to facilitate the slow intravenous administration of such a small quantity of protein solution, the concentrated (25 per cent) solution of human albumin was added to 10 per cent dextrose solution in such proportions that 500 ml. of final solution contained 50 grams of albumin (10 per cent) and 30 grams of glucose (6 per cent). This was administered at a rate of 100 ml. per hour.¹⁷ Control studies were made using 6 per cent dextrose without albumin.

¹⁶ Studies by Dr. Clement Finch on the complement level on 4 patients of this group showed normal values for all but R. S. whose level was steadily and consistently depressed.

SUMMARY OF FINDINGS ON UNTREATED PATIENTS

ELECTROPHORETIC SCHLIEREN
DIAGRAMS

CLINICAL

BLOOD

URINE

	ONSET	B.P. RANGE	EDEMA	WBC (MCU/100 ML.)	CHOLESTEROL (MGU/100 ML.)	TOT PROT. EM (100 ML.)	PROTEIN EM (24 HRS)	RBC
PATIENT JG ♀ AGE 18	5 MO INSIDIOUS EDEMA FOLLOW- ING RESPIRATORY INFECTION	100/60- 145/110	+++	43	1100	28	22	+
PATIENT LI ♂ AGE 43	6 MO INSIDIOUS ALBUMINURIA, 2 MO EDEMA	140/90- 175/120	++	47	1170	38	18	+
PATIENT WH ♂ AGE 42	1 MO EDEMA OLIGURIA SIMUL- TANEOUS WITH RESPIRATORY INFECTION	120/70- 165/100	++	30	755	4.1	19	+
PATIENT DS ♂ AGE 30	18 MO INSIDIOUS ALBUMINURIA, 12 MO EDEMA	110/60- 150/95	++	27	1000	53	11	±
PATIENT KN ♀ AGE 40	5 WKS INSIDIOUS ALBUMINURIA, EDEMA	90/60- 130/70	+	23	430	56	7	+
PATIENT EB ♀ AGE 44	12 MO INSIDIOUS PROTEINURIA, 3 MO EDEMA	125/80- 170/100	+	49	880	41	5	±
PATIENT RS ♂ AGE 29	4 MO INSIDIOUS EDEMA WITH IMPROVEMENT, 3 WKS OLIGURIA, SMOKY URINE	140/90- 175/115	+	61	340	56	12	+++
NORMAL POOLED HUMAN PLASMA								

FIG. 1

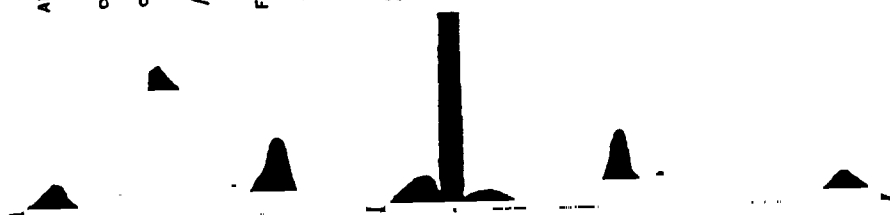
PATIENT J. G.

PLASMA

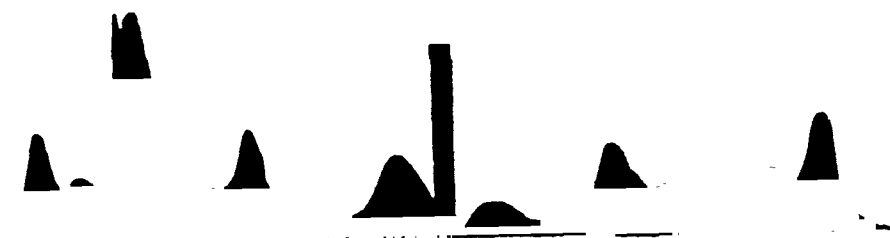
ASCENDING BOUNDARIES DESCENDING BOUNDARIES

ALBUMIN α_1 GLOBULIN α_2 GLOBULIN β GLOBULIN FIBRINOGEN γ GLOBULIN δ ANOMALY ϵ ANOMALY γ GLOBULIN FIBRINOGEN β GLOBULIN α_2 GLOBULIN α_1 GLOBULIN ALBUMIN

FEBRUARY 1944



AUGUST 1944



NOVEMBER 1944

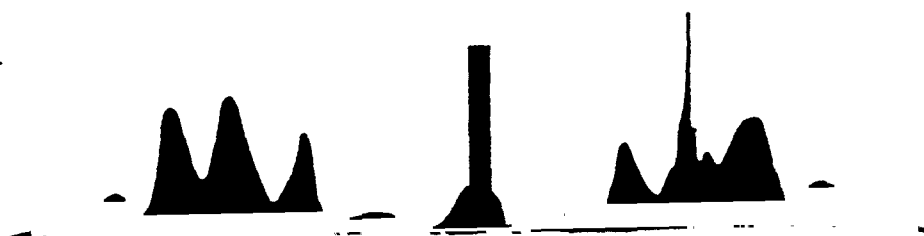


Fig. 2

In 1 patient, R. S., with moderate hypertension, azotemia, and *no edema*, the administration of 50 grams of albumin at 10 grams per hour on 3 successive days resulted in a rise in serum albumin level from 2.5 grams to 3.5 grams per 100 ml. This change was associated with a gain in body

to arrange infusions at times when they would interfere with meals. It was found practical to give the patient an early lunch at 11:30 a.m. and then to give the albumin from 1:00 p.m. to 6:00 p.m. after which dinner was served. It was also convenient to give the evening meal at 5:30 p.m. and administer the albumin from 7:00 p.m. to midnight.

weight, no appreciable diuresis, and a definite increase in blood pressure accompanied by headache, slight nausea, and some cardiac enlargement by x-ray, which receded in the next 5 days. Further treatment was deemed inadvisable.

2. Preliminary experiments

Studies on the effect of a single large dose of albumin in patient J. G. with massive azotemia are illustrated in Figure 3. It is apparent that immediately following the beginning of administration there was a striking increase in urine volume

associated with, though not preceded by, a rise in the rate of excretion of protein, chloride, and phosphorus and to a lesser extent of non-protein nitrogen.

Such experiments showed that albumin in quantities greater than 50 grams daily was not proportionately more effective as a diuretic agent. In adults, quantities of 25 grams or less daily could not be depended upon to induce a diuresis and a positive nitrogen balance. Hence, with a limited supply of albumin available, a standard dose of 50 grams daily was adopted. It is possible that a quantity of albumin which was insufficient to induce a diuresis in an edematous patient might have been adequate to maintain a diuresis once it had been established with a larger dose.

3. Diuretic effect of salt-poor concentrated human albumin administered intravenously

A. A single infusion of 50 grams. In 18 experiments carried out on 7 patients, an average

increase in urine volume of 480 ml. and an average loss of body weight of 0.5 kgm. were observed on the day of infusion. Details of these experiments are presented in Table II. The amount of diuresis, varying from 140 to 1,240 ml., appeared to be roughly correlated with the severity of edema.

In these experiments 500 ml. of fluid were deducted from the patient's constant daily water intake on the day of infusion. That 500 ml. of 6 per cent glucose when infused over a period of 5 hours did not ordinarily induce the diuresis when substituted for an equivalent quantity of water in the diet is indicated by the following experiment made on the patient whose average increase in urine volume on 50 grams of albumin approximated 1,000 ml.

Patient	Urine volume control	Urine volume Rx. glucose intravenously
J. G.	910 ml.	875 ml.

B. Fifty grams daily for 3 days (total 150 grams). An increase in urine volume occurred

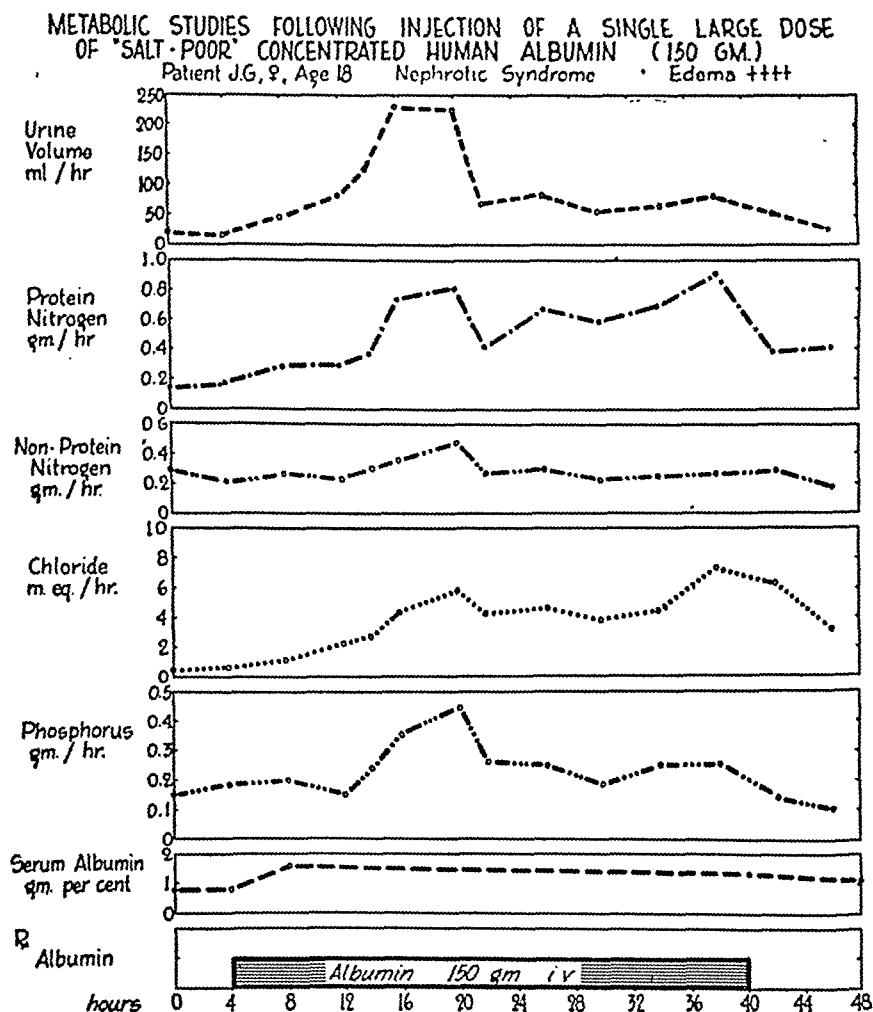


FIG. 3

TABLE II

Effect of a single dose of 50 grams of salt-poor albumin on urine volume in chronic nephritis, constant fluid intake

Patient	Edema	Control urine volume	Urine volume on day of treatment
J. G.	4+ 4+ 4+ 4+	ml. 820	ml. 1720 1920 1630 2060
L. I.	4+	780	1980
W. H.	2+ 2+ 2+ 1+ 1+	2010	2620 3130 2630 2300 2790
D. S.	2+ 2+ 1+ 1+	1600	1850 2480 1220 1820
K. N.	1+	1920	2150
E. B.	1+	2160	1850
R. S.	0 0	2960	3410 3100

during the period of albumin administration in 13 of the 15 experiments carried out on 7 patients. During the control 3-day period, the daily urine volume was 1,750 ml., whereas during the period of albumin therapy the average daily volume was 2,280 ml., yielding an average daily increase of 530 ml. The average daily weight loss was 0.5 kgm. per day or a total of 1.5 kgm. for the 3-day period. Details of such an experiment on patient L. I. are presented in Figure 4. Thus, the average

daily change in urine volume and body weight during the 3-day experiments was comparable to that observed during the 1-day experiment. Again the amount of diuresis appeared roughly correlated with severity of edema; the 2 patients who failed to have an increase in urine volume were R. S., who had no edema clinically, and D. S., who had only barely detectable edema at the time of this particular experiment.

C. *Fifty grams daily for 10 days* (total 500 grams). Five experiments were carried out on 3 patients of which 1, J. G., had massive anasarca, whereas the other 2, D. S. and W. H., had minimal edema during this period. In the first patient, the striking and continued diuresis with average daily weight loss of 0.8 kgm. is illustrated in detail in Figure 5. The courses of the latter 2 patients in whom only slight increases in urine volume over the control periods were observed are presented in Figures 6 and 7.

D. *Fifty grams daily for 22 days and 30 days* (total 1,100 and 1,500 grams respectively). More prolonged courses of albumin therapy were given to 2 patients, J. G. with massive edema, and W. H. at a time when edema was barely detectable. The contrast between their responses is illustrated in Figures 8 and 9.

Patient W. H. showed no diuresis and lost no weight during therapy. However, at the termination of therapy, there occurred a gain of weight of approximately 3 kgm. in 10 days.

Patient J. G., who for months before this experiment had gained weight inexorably (Figure

TABLE III

*Summary of effects of salt-poor concentrated human albumin on urine volume in chronic nephritis**

Dosage of albumin Duration of therapy	Number of experiments	Number of experiments with increased urine volume	Average		
			Urine volume in control period equal to that of therapeutic period	Urine volume in period of therapy	Change in weight in period of therapy
50 grams × 1 day = 50 grams	18	16	ml. 1750	ml. 2230	kgm. - 0.5
50 grams × 3 days = 150 grams	15	13	5250	6840	- 1.5
50 grams × 10 days = 500 grams	5	5	14800	20300	- 2.7
50 grams × 22 days = (edema +) 1100 grams	1	0	44220	45760	0
50 grams × 30 days = (edema ++++) 1500 grams	1	1	24000	37200	-22.0
Total	40	35			

* Five instances in which urine volume was not increased occurred in patients with edema either absent or minimal.



Fig. 4

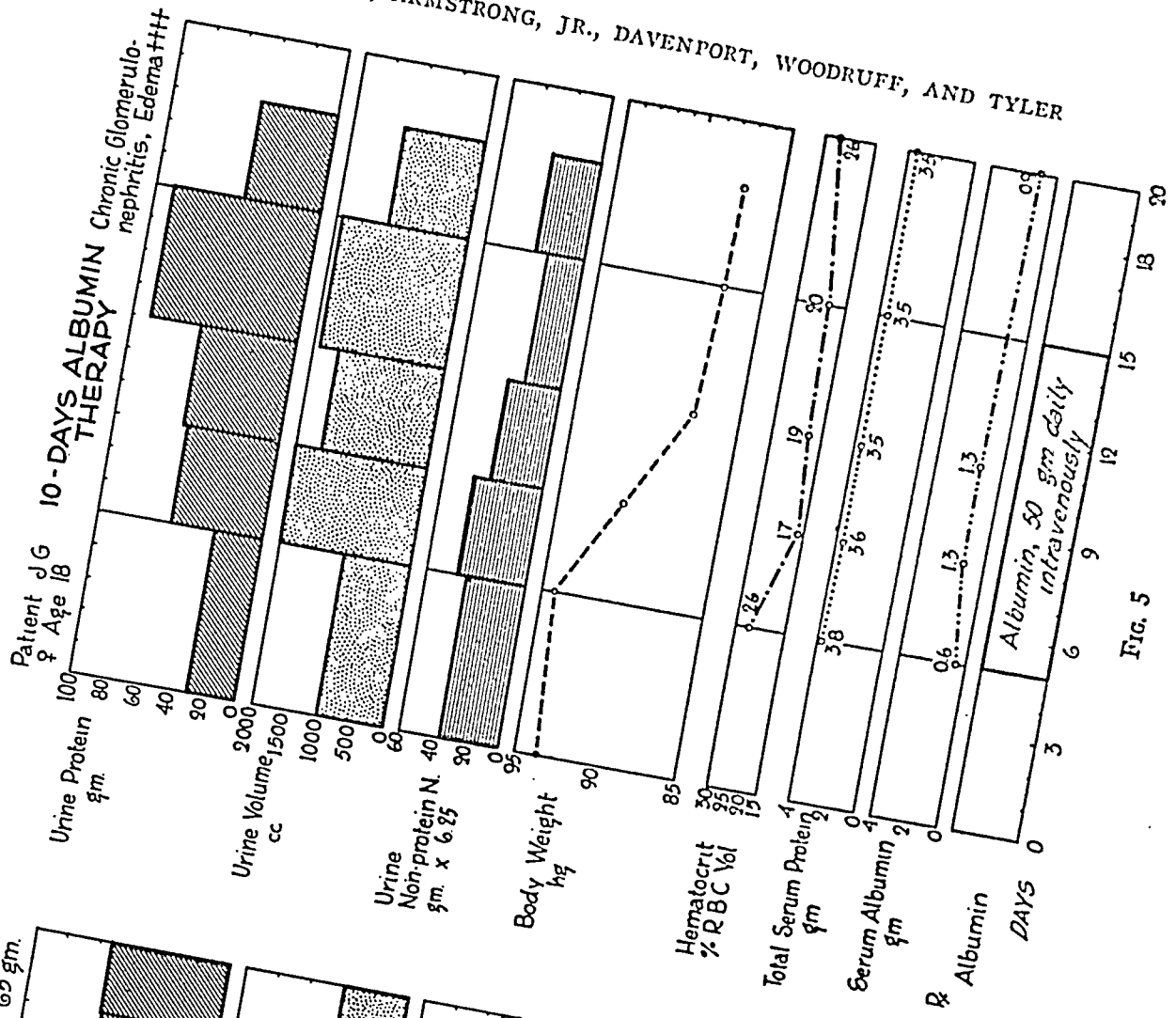


Fig. 5

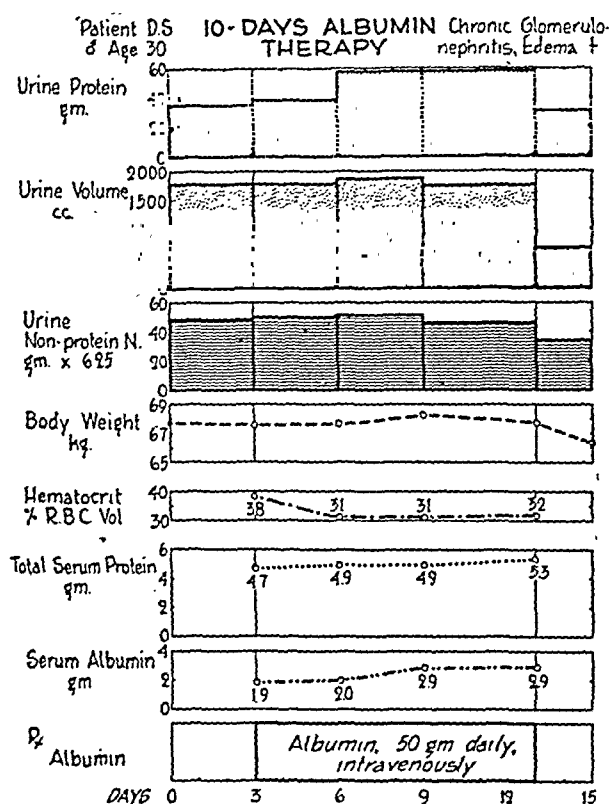


FIG. 6

10), showed an average increase in urinary output over that of her control figure of 1,110 ml. for 30 days and lost 0.76 kgm. daily or a total of 22.9 kgm. during the experiment.

A summary of the observations in the 40 experiments is presented in Table III. It is of interest that the 5 failures to increase urine volume during the administration of salt-poor albumin occurred either in patients with no visible edema or at most minimal edema.

E. Maintenance of weight loss following albumin therapy. In the edematous patients, the maintenance of weight loss appeared as a rough approximation to be related to 2 factors; first, the long-term trend of the patient's weight curve during control periods, and secondly, duration of therapy.

Thus patient J. G. showed the beginning of a weight gain within 24 hours after the diuresis of 3-day albumin administration. After the diuresis of 10-day albumin administration, her weight remained constant for 4 days. After the extensive diuresis during the 30-day administration, her

weight remained constant for a control period of 20 days.¹⁸

In contrast to patient J. G., patient L. I., also edematous, not only maintained his weight loss, but continued to diurese at an accelerated rate following 3-day albumin administration (Figure 4). It is of interest that his weight curve for some weeks previous to therapy had tended slowly downward at the rate of 1 to 2 kgm. a week. Further study will be needed to determine whether, in a patient undergoing slow diuresis under routine therapy, relatively small doses of albumin may precipitate acceleration of weight loss.

4. Effect of salt-poor concentrated human albumin on serum proteins

The serum protein values here presented were obtained by the Howe method occasionally supplemented by electrophoretic analyses¹⁹ on serum taken at 8:00 a.m. following the last albumin treatment, i.e. 8 hours after the end of the infusion.

A. Single injection of 50 grams. Serum protein values were followed in 4 patients who received a single injection of 50 grams of albumin. On the morning after the injection, there appeared to be a slight rise in serum albumin level, a slight decrease in serum globulin level, with little change in total protein concentration (Table IV). The reduction in hematocrit from 33 per cent volume packed cells to 29 per cent suggests that the total circulating albumin was increased appreciably, and that total content of globulin in the circulation was neither greatly increased or decreased and may have been merely diluted. These changes were very transient, moreover, lasting only 24 to 48 hours at most.

¹⁸ At the end of this period, administration of penicillin and removal of an abscessed tooth were followed by a slow spontaneous diuresis.

¹⁹ Electrophoretic analyses (uncorrected, for refractive indices) yielded, as has been observed by previous authors (27, 28), albumin-globulin ratios consistently lower than those obtained by the Howe method. Where simultaneous measurements have been made following albumin administration, the direction and, indeed, the degree of change have as a rule agreed quite closely by the two methods. Notable exceptions have a few isolated instances where little or no albumin increase was revealed by the Howe method following albumin administration, whereas considerable increase was indicated by electrophoresis. Such discrepancies are noted in the tables.

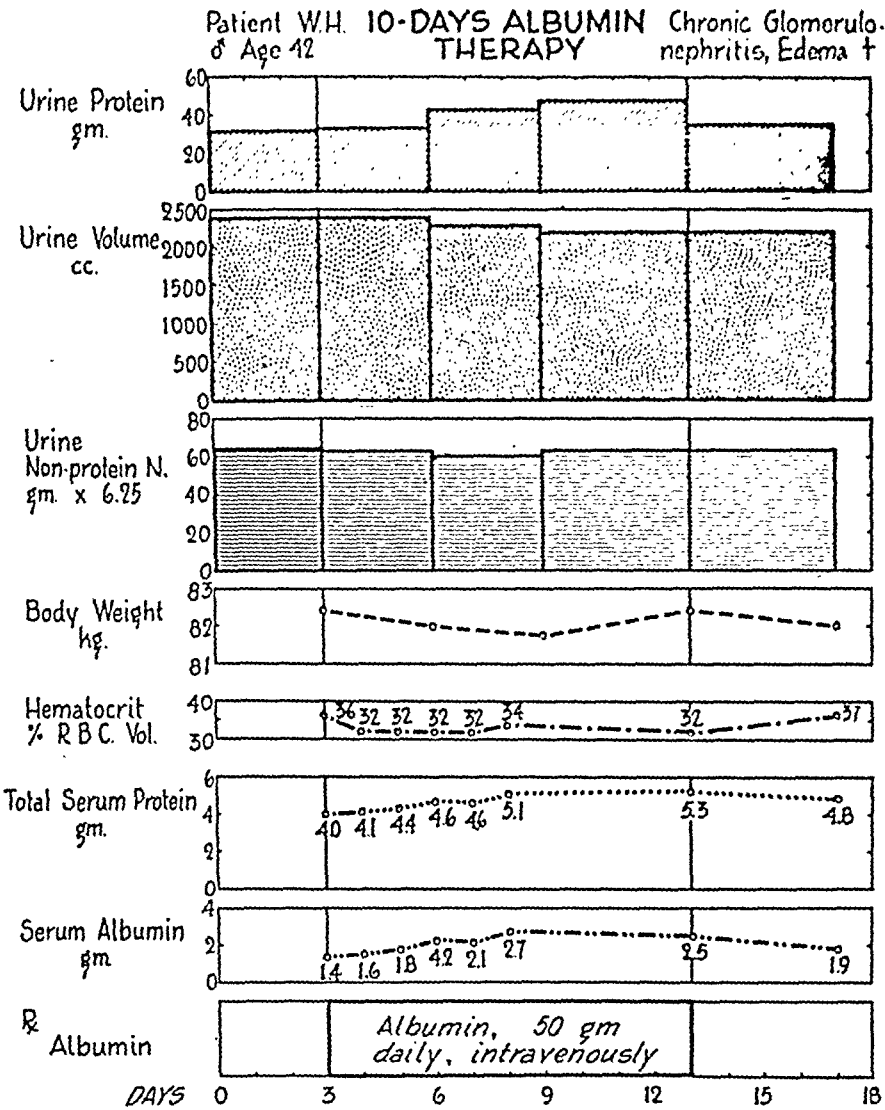


FIG. 7

B. Three daily injections of albumin (total 150 grams). In all 7 patients who received a total of 150 grams of albumin, serum albumin level in-

creased. The average change for the entire group was 0.4 gram per 100 ml. (Table V). In 4 of these patients, some elevation of albumin level persisted for at least 3 weeks following cessation of therapy.

TABLE IV
Changes in serum proteins noted 8 hours after a single infusion of 50 grams of salt-poor concentrated human albumin (Howe method)

Patient	Serum albumin		Serum globulin		Hematocrit (cell volume)	
	Control	Treat-ment	Control	Treat-ment	Control	Treat-ment
	grams per 100 ml.		grams per 100 ml.		per cent	
J. G.	0.6	→ 0.9	3.1	→ 2.5	26	→ 23
W. H.	0.6	→ 1.4	2.6	→ 2.3	31	→ 31
D. S.	1.4	→ 1.6	3.0	→ 2.5	43	→ 33
R. S.	2.4	→ 2.5*	4.1	→ 4.1*	31	→ 28
Average	1.3	→ 1.6	3.2	→ 2.9	33	→ 29

* Electrophoretic evidence indicated a much larger increase in albumin and decrease in globulin in this instance.

C. Ten daily injections of albumin (total 500 grams). In 5 experiments on 3 patients (detailed in Table VI), it was obvious that 10 days of therapy were more effective in raising serum albumin level than either the 1- or 3-day periods. All patients showed increased albumin levels in all experiments, the smallest increase being 0.7 gram per cent, the maximum 1.3 gram per cent, and the average 1.0 gram per cent. In 3 experiments, serum albumin level fell rapidly following the last injection of albumin, although after 3 or 4 days, the levels were still slightly elevated over control figures.

TABLE V

Effect of 3 daily injections of albumin (total 150 grams) on serum proteins (Howe method)

Patient	Serum albumin		Serum globulin		Hematocrit (cell volume)	
	Control	Treatment	Control	Treatment	Control	Treatment
	grams per 100 ml.		grams per 100 ml.		per cent	
J. G.	0.8	→ 1.1*	2.4	→ 2.3*	23	→ 16
L. I.	0.8	→ 0.7	2.6	→ 2.7	43	→ 32
W. H.	0.9	→ 1.4	2.7	→ 2.8	33	→ 29
D. S.	2.2	→ 1.7**	2.0	→ 2.9**	32	→ 30
K. N.	2.8	→ 3.5	3.2	→ 3.1	40	→ 36
E. B.	1.7	→ 2.6	2.4	→ 2.6	40	→ 35
R. S.	2.5	→ 3.5	4.1	→ 3.4	28	→ 26
Average	1.7	→ 2.1	2.8	→ 2.8	34	→ 29

* Electrophoretic analysis, uncorrected, indicated an albumin increase from 0.3 to 1.2 grams per 100 ml., with a corresponding reduction in globulins.

** Electrophoretic analysis, uncorrected, indicated an albumin increase from 1.4 to 2.2 grams per 100 ml., with a corresponding reduction in globulins.

D. Albumin injected for 22 and 30 days (total 1,100 and 1,500 grams respectively). Following the long-term administration to patient J. G. with

TABLE VI

Effect of 10 daily injections of albumin (total 500 grams) on serum proteins (Howe method)

Patient	Serum albumin		Serum globulin		Hematocrit (cell volume)	
	Control	Treatment	Control	Treatment	Control	Treatment
	grams per 100 ml.		grams per 100 ml.		per cent	
J. G.	0.6	→ 1.3	3.2	→ 2.3	26	→ 19
	0.9	→ 2.2	2.6	→ 2.4	26	→ 26
W. H.	1.4	→ 2.5	2.6	→ 2.6	36	→ 32
	1.9	→ 3.0	2.9	→ 2.5	37	→ 35
D. S.	1.9	→ 2.9	2.8	→ 2.4	36	→ 32
Average	1.4	→ 2.4	2.8	→ 2.5	34	→ 29

edema and patient W. H. without edema, it was apparent that the increase in albumin level was not appreciably greater than that observed in the 10-day experiments, nor was the elevated level of albumin maintained following therapy for a longer period of time. Within 10 days after cessation of administration, levels had attained the

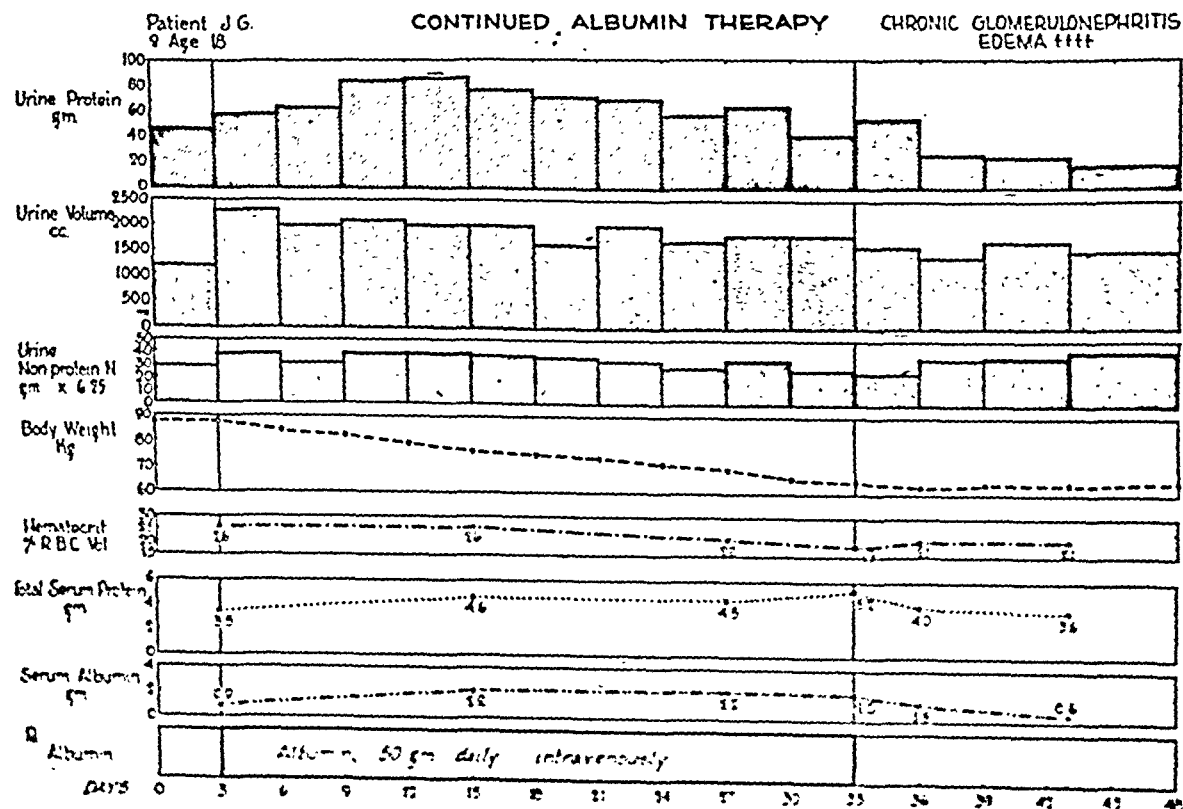


FIG. 8

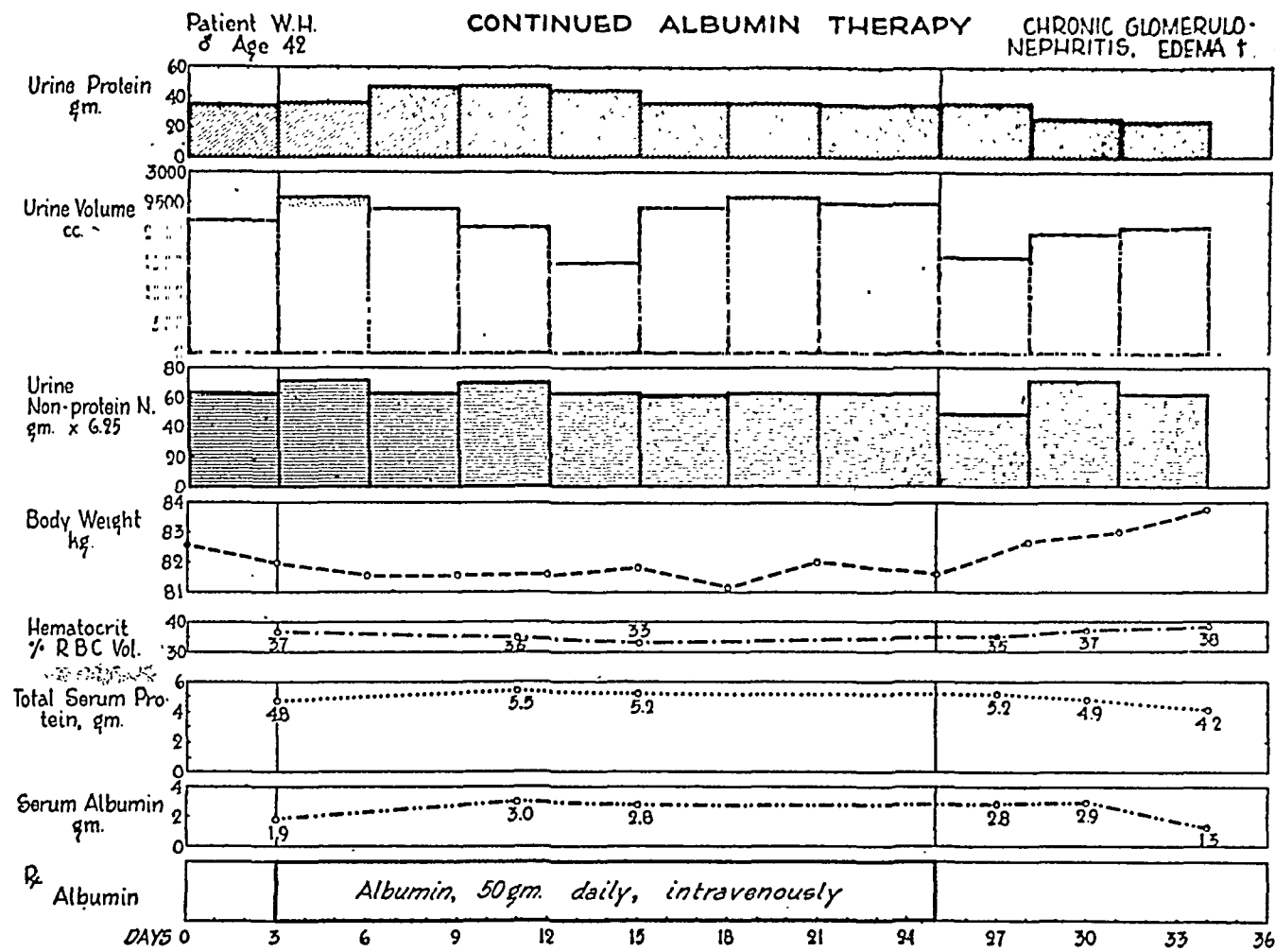


FIG. 9

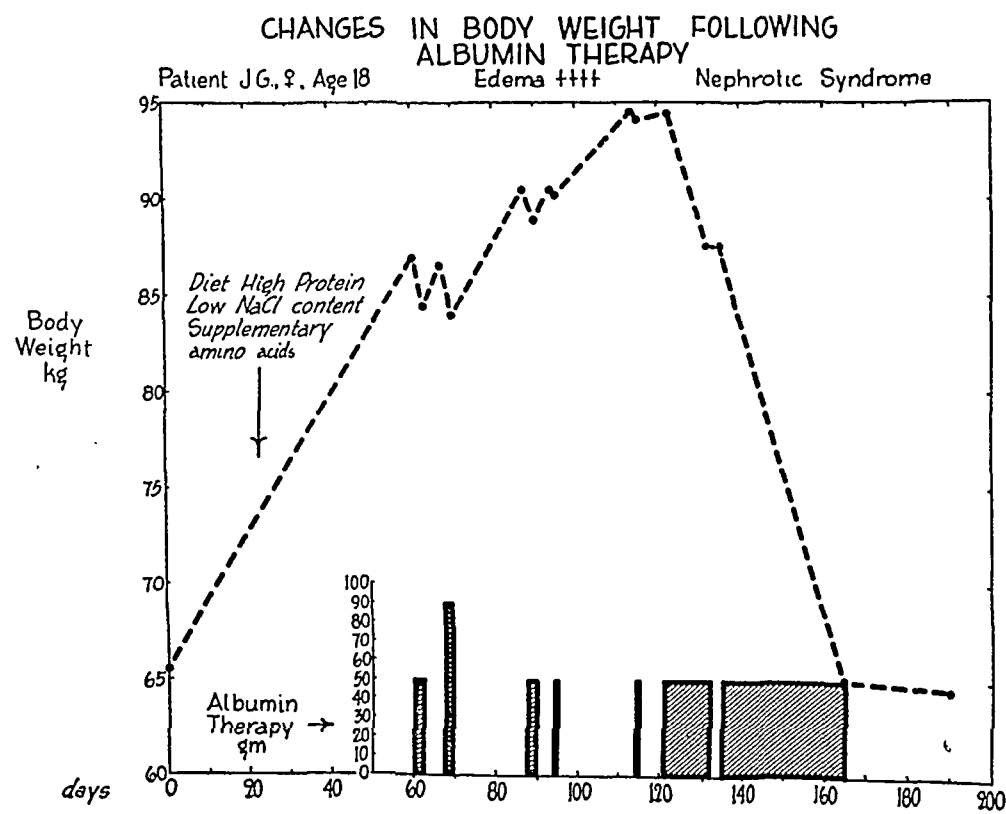


FIG. 10

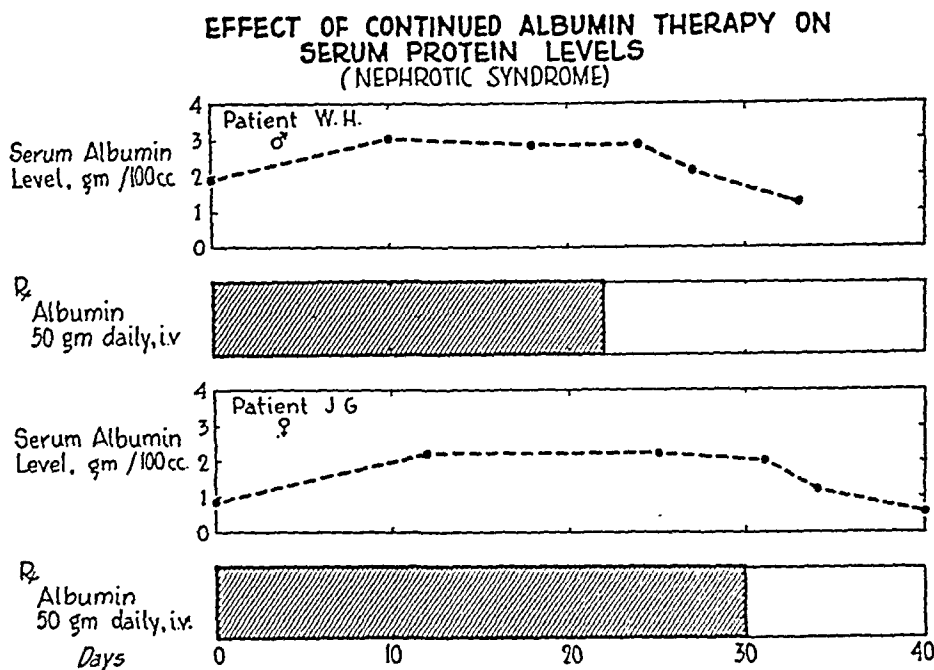


FIG. 11

control values (Figure 11). Details of these experiments are presented in Figures 8 and 9. A summary of the effect of various dosages on serum albumin level is shown in Figure 12.

E. Effect of albumin administration on distribution of electrophoretic components. Beyond an inconstant decrease in ratio of the elevated beta globulin to the total globulins, no significant change in ratios between the various electrophoretic globulin components, either in plasma or urine, could be demonstrated as an immediate result of albumin therapy over the periods previously discussed. This may be seen in Figures 13 and 14 in which are presented electrophoretic schlieren diagrams of patient W. H., whose initial low

gamma globulin and high beta and alpha-2 globulin are characteristic of the nephrotic syndrome, and by contrast, patient R. S., whose high gamma globulin suggested a more acute process. Whereas the relative areas of the albumin peaks increase, the globulin peaks bear approximately the same relation to one another. Moreover, in the patients studied following short-term albumin injections, reversion to control pattern had occurred within 2 to 3 weeks.

Four months after treatment had been discontinued, W. H. presented the identical electrophoretic diagram as that done during the control period. Furthermore, no change in clinical condition or urine sediment was noted during this period. The gradual reversion in the electrophoretic pattern of patient J. G. toward normal, although it took place following prolonged albumin administration, cannot unequivocally be ascribed thereto. It is of interest that the reversion in electrophoretic pattern was associated with striking clinical improvement and a decrease in proteinuria.

F. The effect of albumin administration on total circulating globulin. Estimations of changes of total circulating globulin calculated from changes in hematocrit and plasma protein distribution by both the Howe and the electrophoretic methods

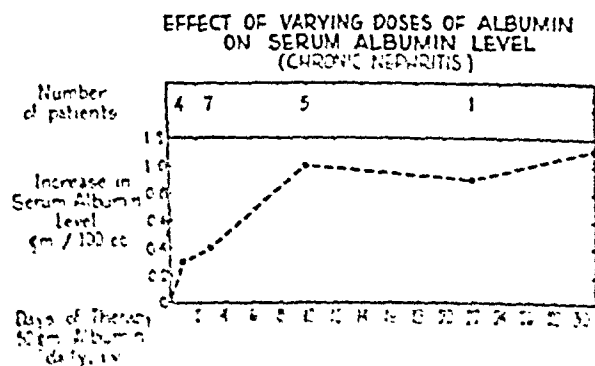


FIG. 12

were inconstant in direction and well within the considerable limits of error which the methods involve (Table VII). As in the instance of the 3-day injection, the fact that the total globulin content was neither greatly increased nor decreased suggests that the effect of albumin administration was merely a dilution of the globulins. It should be pointed out that the methods employed are far too insensitive to detect such small increases of total circulating globulin as have been described immediately following a single injection of 25 grams (15).

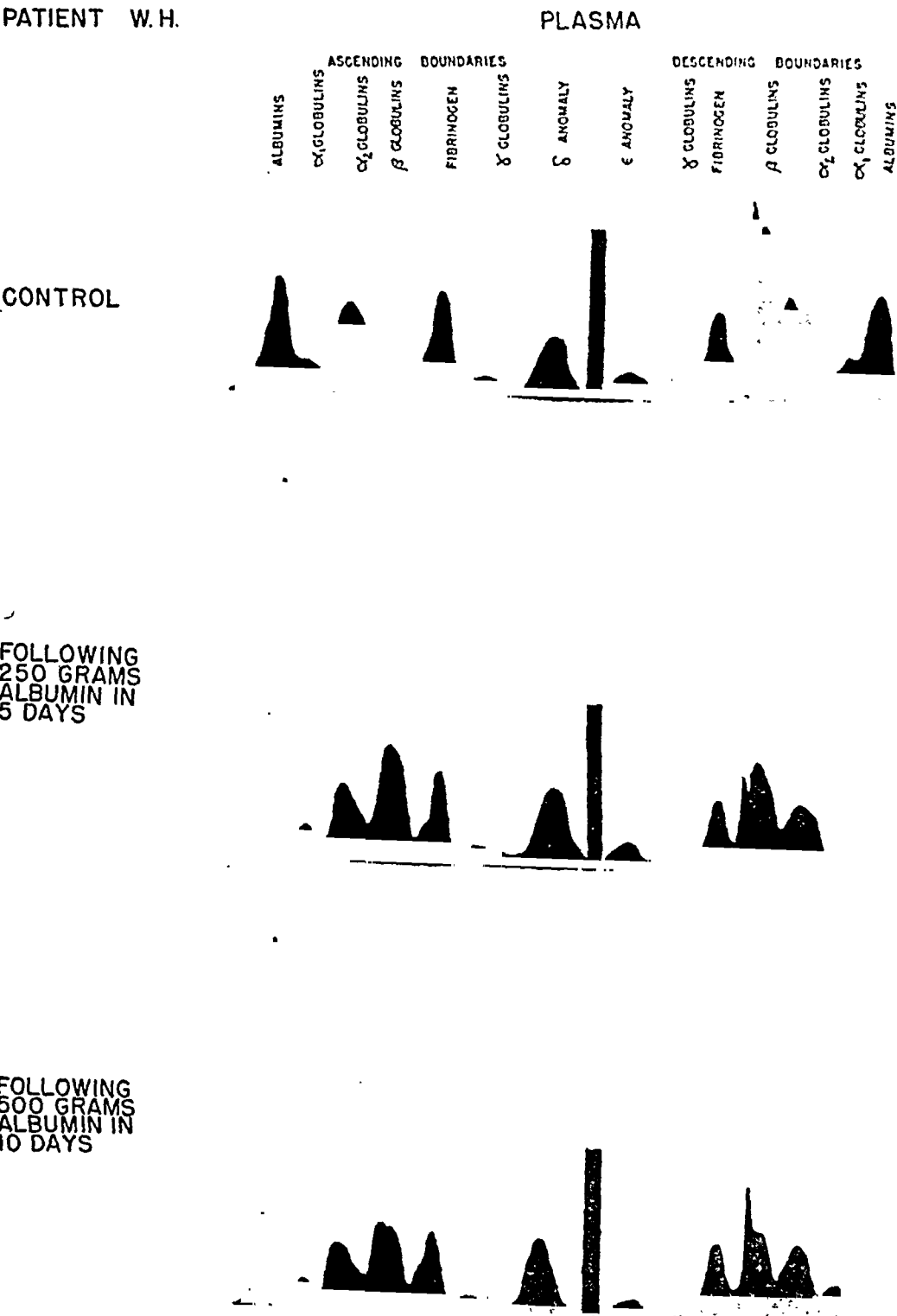


FIG. 13

TABLE VII

Comparison of estimates of effect of albumin administration on amount of circulating albumin and globulin by uncorrected electrophoresis and by the Howe method

Patient	Experiment		Uncorrected electrophoresis	Howe salting out	Uncorrected electrophoresis	Howe salting out
			grams of albumin per 100 ml. RBC		grams of globulin per 100 ml. RBC	
W. H.	500 grams in 10 days	Control	1.7	2.5	5.3	4.7
		Following albumin	4.7	5.3	5.6	5.7
	1100 grams in 22 days	Control	2.7	3.2	5.4	4.9
		Following albumin	5.2	5.7	5.4	4.9
D. S.	150 grams in 3 days	Control	3.0	4.7	6.0	4.2
		Following albumin	5.0	3.9	5.6	6.7
	500 grams in 10 days	Control	2.6	3.2	5.0	4.5
		Following albumin	6.0	6.0	5.3	5.0
R. S.	150 grams in 3 days	Control	5.3	6.4	11.4	10.3
		Following albumin	8.6	10.0	11.0	9.7

5. Effect of albumin administration on plasma volume

Whereas estimated increases in plasma volume per gram increase in circulating albumin showed wide spread (coefficient of variation 28 per cent) owing to the errors inherent in the nature of the data available for calculation, it is of interest that the average value was fairly close to the value predicted from osmotic pressure measurements. (11).²⁰ It is of the same order of magnitude as that derived from experiments on injection of concentrated albumin following acute blood loss. (29).

Average plasma protein concentration	Average estimated increase in plasma volume (ml.) per gram increase circulating albumin	Predicted increase in plasma volume (ml.) per gram increase circulating albumin
grams per 100 ml. 4.9	19	20

²⁰ In that the value of Scatchard and his associates (Figure 3 of their paper (6)), is based on iso-osmotic addition of protein to a system, it is not strictly comparable to measurements on these patients where albumin administration in all instances yielded increases in albumin concentration in addition to increases in plasma volume. Correction of Scatchard's figure for this different situation would involve a small revision downward, the size of which is insignificant in comparison with the size of the errors in computing plasma volume changes from hematocrit and hemoglobin data.

It also is of interest that patient J. G., whose plasma proteins were significantly lower than the average appeared to show with some consistency, as would be predicted from osmotic pressure data, larger increases in plasma volume than the average.

6. Studies on nitrogen balance

A. *A single injection of albumin (50 grams).* Protein excretion increased on the day of albumin therapy by approximately 7 grams in the 7 patients studied. Studies in 4 patients (J. G., D. S., W. H., and R. S.) for 3 successive days revealed that an additional 21 grams was excreted before equilibrium was attained. Striking differences in protein excretion were observed in different patients (Table VIII). The largest loss of protein occurred in the patient with the greatest initial

TABLE VIII
Urinary protein excretion following a single injection of 50 grams of albumin

Patient	Control grams per 24 hrs.	Day of treatment grams per 24 hrs.
J. G.	24	32
L. I.	18	22
W. H.	19	26
D. S.	32	39
K. N.	8	6
E. B.	7	13
R. S.	18	24
Average	23	22

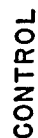
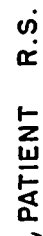
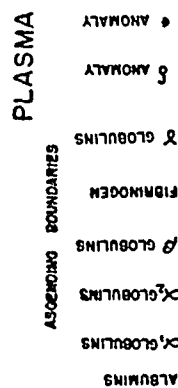
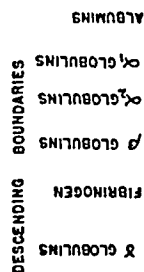
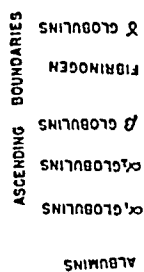
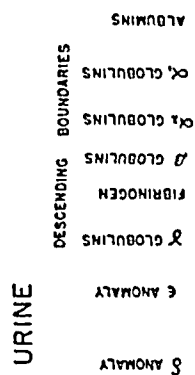


FIG. 14

proteinuria (J. G.). Such a correlation did not appear to hold for the rest of the patients. Although proteinuria increased, nonprotein nitrogen decreased; and balance studies indicated a total net gain of nitrogen of 4.9 grams, equivalent to 31 grams of protein or approximately 60 per cent of the injected dose of albumin.

B. *Three daily injections of albumin* (total 150 grams). Studies on 6 patients indicated an increase in proteinuria during the 3 days of treatment and for at least 3 days after treatment was discontinued in 5 of the group (Table IX). The increase in protein excretion during the period of treatment amounted to approximately 16 grams daily (32 per cent of injected albumin), and an

TABLE IX

Urinary protein excretion following 3 days of albumin therapy (total 150 grams)

Patient	Control	Treatment			Following treatment		
		Day 1	Day 2	Day 3	First day	Second day	Third day
		<i>grams per 24 hours</i>					
J. G.	24	39	59	56	48	33	27
L. I.	18	32	60	39	40	36	36
W. H.	19	24	38	38	40	39	24
D. S.	12	17	44	52	53	16	19
K. N.	8	6	6	6	8	7	
R. S.	15	16	18	28	19	29	23
Average	16	22	37	37	35	27	23

additional 12 grams daily was excreted during the subsequent 3 days. Thus, approximately 56 per cent of the albumin injected was excreted during the period of injection. Nitrogen balance studies indicated a slight overall reduction in nonprotein nitrogen excretion with a resulting positive balance during the entire experiment of 12.9 grams of nitrogen equivalent to 81 grams of protein or approximately 54 per cent of the injected protein.

In 3 experiments on 2 patients no increase in average daily globulin output was observed on albumin dosage of this magnitude (Table X). This both confirms and extends the identical finding following injection of a single 25-gram dose (15).

C. *Ten daily injections of albumin* (total 500 grams). Three patients were studied during and following 10 days of albumin therapy. Albumin excretion increased by an average of 36 grams daily during the 10-day period of therapy (Table

TABLE X

Excretion of globulins following albumin administration

Patient	Amount of albumin	Number of days	Average 24-hour urine output		Average 24-hour globulin output	
			Control	Treated	Control	Treated
	<i>grams</i>		<i>ml.</i>		<i>grams</i>	
J. G.	150	2	820	1840	8.7	7.1
	178	3	820	1700	7.5	8.4
R. S.	150	3	2960	2930	5.7	5.2

XI). During the 3 days following cessation of treatment an increased excretion of 18 grams (average) per day was noted. Approximately 80 per cent of the injected albumin was thus excreted during the experiment. Nitrogen balance studies, however, indicated a slight overall reduction in nonprotein nitrogen excretion with a consequent average nitrogen retention of 20 grams of nitrogen equivalent to 124 grams of protein during the experiment. This represented a retention of approximately 25 per cent of the injected protein.

D. *Longer continued studies—22 and 30 daily injections of albumin* (total 1,100 and 1,500 grams respectively). The results of these experiments are summarized in Figures 7 and 8. Patient W. H. excreted an excess of 3.4 grams nitrogen daily (22 grams of protein) during the 22-day experiment. During a 9-day period following withdrawal of therapy, excess proteinuria amounted to 11 grams daily. This total increase in protein excretion amounted to 580 grams. Nitrogen balance indicated a total positive balance of 52 grams of nitrogen or 327 grams of protein equivalent to 30 per cent of injected protein.

Patient J. G. excreted 42.3 grams of protein daily above her control level of excretion during

TABLE XI

Urinary protein excretion following 10 days of albumin therapy (total 500 grams)

Patient	Control	Treatment				Following treatment 3 days
	3-day period	1-3 days	3-5 days	5-10 days	10-15 days	
		<i>grams per 24 hours</i>				
J. G.	24	56	55	59	46	
W. H.	19	34	43	47	36	
D. S.	12	39	34	54	32	
Average	19	43	44	51	38	

the 30-day period of treatment, and 10.3 grams daily during the 10-day period following cessation of therapy. This represented a total loss of approximately 1,370 grams of protein or 90 per cent of the quantity of injected albumin. Nitrogen balance studies, however, indicated a retention of 1.6 grams of nitrogen daily, or 10 grams of protein, with a negative balance of 2.7 grams daily for 3 days after treatment was discontinued, balance being established after the third day. Summary of this indicates a total overall retention of approximately 40 grams of nitrogen or 250 grams of protein during the experiment, or approximately 16 per cent of injected protein.

A summary of all nitrogen balance studies is presented in Table XII.

TABLE XII

Summary of nitrogen balance studies in patients with chronic nephritis treated with salt-poor concentrated human albumin intravenously

Number of patients	Group of experiments (albumin)	Average protein retention	Total injected protein retained
	grams	grams	per cent
4	50	30	60
6	150	81	54
3	500	124	25
1	1100	327	30
1	1500	250	16

7. Follow-up studies

The response of 2 patients to the injection of 150 grams of albumin over 3 days was checked some 6 months after the original studies. The first patient, J. G., had lost her massive anasarca, her serum albumin level had risen from 0.8 to 1.9 grams per cent, and her daily spontaneous proteinuria had decreased from 24 to 13 grams. In this improved condition 150 grams of albumin occasioned a rise of serum albumin level to 3.7 grams per cent as against the rise to 1.1 gram per cent noted during the initial study. Sixty-two per cent of the injected nitrogen was retained as against an initial 21 per cent.

Patient K. N., originally in a far less severe nephrotic state, showed no essential change in clinical condition at the time of follow-up injection. Her response to 150 grams of albumin was almost identical to her initial response both from the standpoint of change of serum albumin level and retention of protein nitrogen.

8. Effect of other forms of therapy on this group of patients

A. Mercupurin. Over a period of 14 days, patient J. G. was given 4 injections (totaling 7.5 ml.) of mercupurin, in 3 instances preceded by 8 grams of ammonium chloride. At this time, her weight curve was showing a gain of 2.5 kgm. a week (Figure 10). Although the urine volume increased some 300 ml. per 24 hours over the average control value following each injection, during the period of therapy the patient continued to gain weight at a rate identical with the control period.

*B. Amino acids.*²¹ *Patient J. G.* During the period when this patient's already massive edema was increasing at a rate of 2.5 kgm. a week, she received approximately 70 grams of amino acids daily by mouth for 10 days. Although the average daily urine volume increased by approximately 300 ml. over that of the control period, weight gain continued at the same rate. There was no significant change in total protein or albumin levels.

Patient R. S. At a time when his edema was barely detectable and his basal weight curve was falling 1 kgm. a week, the patient received approximately 60 grams of amino acids a day orally for 8 days. During this period, he lost 3 kgm. He gained 1 kgm. in the week following cessation of therapy after which his weight curve remained essentially flat. There was no change in the plasma proteins.

Patient W. H. In contrast to patient R. S., on essentially the same daily dosage of amino acids over a period of 30 days, W. H. gained 1.5 kgm. per week, although during the preceding control period he had lost 3 kgm. a week. His moderate edema did not decrease.

Patient D. S. A similar increase in weight, although slightly less in amount, occurred in patient

²¹ The oral and intravenous preparations of Frederick Stearns and Company were used. The oral preparation contains 2.8 per cent nitrogen; 280 ml. contain the same amount of nitrogen as 50 grams of albumin. The intravenous preparation contains 2 per cent nitrogen; 400 ml. are equivalent in nitrogen to 50 grams of albumin. The use of these preparations increased only slightly the sodium intake of the basal regimen, for they contain approximately 0.130 gram sodium (expressed as sodium chloride) per 100 ml.

D. S. during both 15 days of oral amino acids therapy at this identical dosage, and 9 days of intravenous amino acids administration at a nitrogen dosage equivalent to 37.5 grams of albumin a day. During control periods for both these experiments, the patient's basal weight curve decreased at a rate of approximately 1 kgm. a week. During both periods of therapy the average daily urinary volume decreased by approximately 300 ml. over that of the control periods. In neither experiment was there a significant change in serum total protein nor albumin. During the intravenous therapy, N.P.N. excretion increased from a control average of 12.5 to 16.2 grams daily, and protein excretion from 7.5 to 12.5 grams daily. Less than 0.5 per cent of amino acids was excreted in the urine as such. During the 10-day period of therapy and the ensuing 3 days, however, approximately 70 per cent of the injected nitrogen was excreted, yielding a retention of 30 per cent, a figure comparable to that encountered with a dose of albumin of similar magnitude in this patient.

C. *Urea*. The anorexia which so frequently attends the nephrotic syndrome made the oral administration of this substance impossible in the instance of patient J. G. and limited the tolerated dosage in certain other patients.

Of the group, patients L. I., W. H., and D. S., whose edema ranged from minimal to moderate, were able to ingest a dose of approximately 22 grams a day, in the first instance for 9 days, in the latter instances for 22 days. In no case was there an increase in urine output over the control values. In the first 2 patients the weight curves remained flat; in the third patient there was a weight loss totaling 2 kgm. There was no significant effect on the plasma proteins.

A second period of urea administration at a dosage of 90 grams a day was instituted in patient D. S. shortly after his 10-day period of albumin therapy. Whereas during the periods of control, albumin therapy, and urea at a rate of 20 grams a day his weight curve had remained flat, on the larger dosage, he lost weight at the rate of 3 kgm. a week, and his urine output increased over that of the control period by approximately 400 ml. a day. In the 3 days following cessation of urea, the patient regained 3 kgm. Weight loss again occurred at the same rate on the resumption of

90 grams of urea a day. A comparable rate of diuresis was observed over a shorter period in patient R. S. at a time when he had no detectable edema.

This small number of observations suggests that in patients with minimal edema in whom albumin has been a relatively ineffectual diuretic, urea in moderate dosage is likewise without effect, but in large amounts may induce diuresis and maintain it during the period of administration.

DISCUSSION

This study of the use of salt-poor human serum albumin in glomerulonephritis represents a step in a systematic program aimed at the study of the effect of the administration of purified plasma protein fractions, characterized both with respect to physical-chemical properties and in so far as possible to physiological function, in those conditions in which the normal equilibrium state of these proteins is known to be altered.

Whereas the use of a molecule of different characteristics (for example, of similar diameter but increased length yielding increased retention in the blood stream (30) may ultimately prove more effective, salt-poor albumin has several advantages over the various substances of high molecular weight that have been used in attempts to effect diuresis and positive nitrogen balance in patients with low total proteins and a high degree of albuminuria. It is a native protein. Although low in sodium, it shares with normal plasma or serum the fact that its amino acid composition gives it a nutritional capacity approximately equal to that of whole plasma (31) and superior to that of such incomplete proteins as gelatin (32). The reaction rate following injection of normal human serum albumin in our experience is even less than that of banked plasma. Its use in large quantities does not carry the same risk of liver deposition and subsequent impairment of albumin synthesis such as has been reported both experimentally and clinically with atacia (33).

It has been a frequent finding of many workers endeavoring to treat nephrotic edema with solutions of high molecular weight substances that administration of atacia resulted in no prolonged rise in plasma colloid osmotic pressure, nor did administration of plasma or serum yield prolonged rises in serum protein levels (2 to 4, 34).

Such observations have led to many interpretations of the mechanism of the irregularly occurring diureses encountered with these substances. Following a suggestion by Peters(35), evidence was presented for a correlation between diuresis and increase in plasma volume rather than an increase in colloid osmotic pressure (36, 2). Although the possibility of this mechanism found corroboration in studies of urine output following plasma infusion into normal dogs (37), increases in blood volume were observed in nephrotics, following plasma therapy where no diuresis occurred (3). Again, whereas an increased chloride output has been described (34), the chloride loss during diuresis following acacia infusion was roughly equal to the amount of chloride in the injected acacia solution plus that of the excreted edema fluid (38).

Finally, the unchanging serum protein level at the onset of the diureses produced by plasma was reminiscent of the spontaneous diureses of nephrosis and suggested that in addition to osmotic action "serum . . . supplies some . . . substances which set off the patient's own mechanism of diuresis" (1). Hence, appraisal of therapeutic efficacy was based on an increased incidence over the expected of a diuresis of this type.

Indeed, a study of the course of diureses reported following administration of serum, plasma, and acacia reveals that in many instances it further resembles the spontaneous diuresis in that it persists long after the period of administration of colloid substances (39).²²

The diureses following salt-poor albumin appear to differ from those reported with other substances in the time relation between administration and weight loss. In no instance did administration of albumin provoke the type of spontaneous diuresis which long outlasts the period of therapy. The average daily weight loss on uniform daily dosage was approximately the same for periods of therapy varying between 1 and 30 days. This weight loss appeared to be superimposed on the patient's basal weight curve. Thus, in patient J. G. whose basal weight curve over 2 months before therapy showed an average gain of

10 kgm. a month, short periods of therapy with attendant diureses ultimately were followed by continued weight gain. After the loss of 20 kgm. during the 30-day administration, the patient, although still strikingly edematous, maintained a level weight for 3 weeks. The significance of this level weight curve is emphasized by the subsequent spontaneous diureses in the absence of any albumin which began immediately following removal of an abscessed tooth.

In this group of patients the correlation between extent of diuresis and the extent of the patient's edema was striking. The data set forth here do not elucidate the mechanism. Extent of diuresis showed no constant correlation with either increase in serum albumin level or in total circulating albumin. The estimated increases in plasma volume per gram increase in circulating albumin following treatment were but little larger in the edematous patients who showed good diureses than in those with little edema whose weight curves remained flat. The fact that the average daily weight loss per gram albumin was as great on the first as on the third day of a course of treatment, although the average quantity of excreted protein was far less on the first day, excluded any constant correlation with total protein excretion.²³ Nor could diuresis be related to average urinary protein concentration.²⁴

From the correlation between the extent of edema and the diuresis produced by salt-poor al-

²³ This is shown by examination of the charts of J. G. and D. S. between the third or sixth day of their 10-day period of therapy (Figures 5 and 6). Although patient J. G. showed a typical diuresis and D. S. did not, they both excreted an average of 56 grams of protein per day. Moreover, the fact that no temporary increase in urinary protein concentration preceded diuresis following the injection of a single dose of salt-poor albumin in patient J. G. (Figure 3) fails to provide evidence that the presence of high protein concentration in the tubule initiates diuresis. In this connection, it is of interest that in cirrhotic patients (40) who have no proteinuria, the same correlation between occurrence of diuresis and extent of edema would appear to hold.

²⁴ The average urinary protein concentration of patient J. G. during the initial 10 days of her 30-day period was 2.8 grams per cent. At this time she was losing weight at a rate of 0.76 kgm. per day. During the last 6 days in patient D. S.' 10-day period of treatment, his average urinary protein concentration was 3.3 grams per cent at which time the patient having little edema had no change in weight.

²² The diuresis illustrated by Landis (38) taking place during the administration of about 20 grams of acacia a day for 8 days resembles more the diuresis obtained with albumin.

bumin treatment, both the values and the limitations of this form of therapy in the various stages of chronic Bright's disease emerge.

The greatest use would appear to be in the extreme nephrotic stage. In the edema-free patient, tending toward a fixation of specific gravity, nitrogen retention and hypertension, its value is less and occasionally its use may be contraindicated from the cardiovascular standpoint by reason of its ability to produce and maintain striking rises in blood volume.

Indeed, it is in extreme nephrosis, the edema of which is notoriously quite resistant to mercurial diuretics, in which administration of amino acids has no constant effect, and in which anorexia often prohibits oral urea in large doses, that an agent which will induce and maintain a diuresis may occasionally give dramatic symptomatic relief to a desperately sick patient.

It is important to emphasize the basal dietary regimen of these patients in evaluating the ability of albumin to maintain diuresis and positive nitrogen balance. Had not these patients been very close to positive nitrogen balance on the dietary intake alone, larger quantities of albumin may well have been required to induce the significantly positive nitrogen balance attained and the feeling of well-being that appeared to accompany that state. Likewise, it is obvious that the rigid restriction of salt is important in the maintenance of diuresis with salt-poor albumin, particularly in view of the possibility that salt-poor albumin might have succeeded in the previously reported instances (5) where high-salt albumin has failed in attainment of diuresis.

No evidence emerges from this study that the diuresis or positive nitrogen balance induced by salt-poor albumin results in any change in the natural history of the disease process. Clinical follow-up studies have shown no deleterious changes in renal function ascribable to the relatively large doses of albumin.²² This is in agree-

²² The work of Bailey and Hawn (41) on bovine albumin, together with the correlation noted by Blackman and Davis (42) of severe globulinuria with progressive renal damage, would not lead to the expectation of such tubular changes following normal human serum albumin administration as have been described by Huser (43) following the administration of certain forms of purified protein and of egg albumin in dogs, and by Sorenson (44) following

ment with other findings which revealed an instance of complete recovery with absence of proteinuria in an 8-year-old nephrotic boy who had received over 600 grams of high-salt normal human serum albumin in a period of 30 days (5).

SUMMARY AND CONCLUSIONS

Salt-poor concentrated human serum albumin has been administered intravenously for periods varying between 1 and 30 days at a dosage of 50 grams a day and a rate of 10 grams an hour to a group of 7 patients in several stages of chronic nephritis maintained on a diet adequate in calories, containing 80 to 125 grams of protein daily and low in sodium chloride.

The therapeutic value of salt-poor albumin varied with the stage of the disease. In patients with edema, low serum proteins, and absent to moderate hypertension and nitrogen retention, albumin in this dosage was a safe agent in increasing the serum albumin level and in inducing positive nitrogen balance.

The diuretic effect of salt-poor albumin administration was most striking in the severe nephrotic state with massive edema. Control studies both with amino acid mixtures administered by mouth and intravenously in comparable quantities and with mercupurin yielded no diuresis under these circumstances.

Unlike the diureses that have been reported as initiated by a wide variety of therapies and which resemble the spontaneous diureses of the nephrotic state, the diureses following albumin proceeded at a constant rate during the period of administration only and stopped at the end of it.

Following short periods of therapy (10 days or less), the ability to maintain weight loss was roughly related to the slope and direction of the patient's weight curve on basal regimen. In the one instance of prolonged (30-day) therapy in nephrosis, a previously rapidly ascending weight curve became flat following diuresis of 29 mm. although the patient was still edematous. In that

ing absorption and storage of food as intact units and conjugated proteins in the open spaces of nodules. The only direct evidence on this point is provided by the histological examination of the kidneys of L. I. in this series of patients who died of uremia some 6 months after the last albumin treatment. No changes specifically referable to therapy were found.

such a change in basal weight curve is consistent with the spontaneous course of the disease, the significance of this observation as related to salt-poor albumin therapy can only emerge after far wider clinical use.

In contrast to its effect on nephrotic anasarca, the diuretic effect of salt-poor albumin in the presence of minimal edema was slight. Although control studies showed that both amino acid mixtures in comparable quantities and urea (20 grams daily) were likewise ineffective, massive doses of urea (90 grams a day), when tolerated, appeared to induce further diuresis and weight loss of a transient character.

Albumin administration in the presence of severe hypertension, nitrogen retention, and in the absence of edema appeared contraindicated owing to its efficacy in rapidly increasing blood volume beyond the tolerance of the cardiovascular system.

Although in this study salt-poor albumin appeared to have no influence on the natural history of the disease process, in a condition as variable as chronic glomerulonephritis, observations far more extensive, both in duration and in range of clinical material, are necessary to determine this point. The symptomatic benefits here reported in the nephrotic state well warrant such observations.

CASE HISTORIES AND INITIAL CLINICAL FINDINGS

1. J. G. (M-65757), an 18-year-old white school girl, was admitted to the Peter Bent Brigham Hospital on January 24, 1944, because of generalized edema which had progressed to massive anasarca over the past 5 months.

Her health had been excellent with the exception of measles, mumps, and scarlet fever without known complications.

The present illness began in August, 1943, with the onset of ankle edema associated with a head cold. Following this, she felt well and was active, but the edema increased, and she began to note progressive swelling of her abdomen. There were no urinary symptoms beyond the fact that during her rapid weight gain, she noted her urine volume to be unusually small. In the month preceding admission she had anorexia, flatulence, and occasional abdominal cramps.

Physical examination: Temperature 98.6° F., pulse 80, respirations 18, blood pressure 120/76 mm. Hg. A well-developed and -nourished young girl showed marked general pallor and puffiness of the face together with striking soft pitting edema beginning at the mid-abdomen and extending down the lower extremities.

Fundi were not remarkable. Pharynx was normal. There was evidence of fluid in both pleural cavities and the abdomen showed shifting dullness and an easily

demonstrable fluid wave. The heart was not remarkable. The remainder of the examination was noncontributory.

Laboratory data: Blood Hinton negative. Urine, serial specimens: Specific gravity varied between 1.020 and 1.036, protein 4+ (22 grams in 24 hours), sugar negative, centrifuged sediment—red cells varied between 2 and 45 per high power field, white cells varied between 1 and 10 per high power field, casts varied between 1 and 10 per low power field. Blood: Red cells 4,000,000 per c.mm., hematocrit 35, sedimentation rate 61 mm. per hour, white cells 12,000 per c.mm. with 78 per cent neutrophils. Blood chemistry: Urea nitrogen 28 mgm. per cent, total protein 2.8 grams per cent, cholesterol 1,100 mgm. per cent, chlorides 107 m.eq. per liter, carbon dioxide combining power 28 m.M. per liter. Vital capacity 1,500 ml. Electrocardiogram: Low EMF with a P-R interval of 0.20 seconds.

X-ray examinations: Chest: Fluid at both bases and a heart normal in size and shape. Sinuses: negative.

2. L. I. (M-66634), a 43-year-old white male watchman, was admitted to the Peter Bent Brigham Hospital on June 23, 1944, because of generalized edema of 2 months' duration.

The patient had had uncomplicated scarlet fever at the age of 7 without any nephritic sequelae. For at least 6 years before entry, he had suffered from mild bronchial asthma.

Although asymptomatic, a routine urine examination 6 months prior to admission disclosed massive albuminuria. Two months prior to admission, the onset of generalized edema, unassociated with any noticed infection, caused him to consult his family doctor who advised bed rest, thyroid, and a high protein diet. Despite a period of transitory improvement, edema progressed and nausea, anorexia, and constipation appeared.

Physical examination: Temperature 98.0° F., pulse 84, respirations 20, blood pressure 160/110 mm. Hg. A pale moderately obese man presented generalized edema of his legs, genitalia, and to a lesser degree of his abdomen. Fundi showed irregularities in the calibre of the vessels and one small area of exudate above the right disc. The pharynx was red. The tonsils were large and red; no pus was observed. Many asthmatic squeaks and fine basal rales were heard over both lung fields. The heart was not enlarged; rhythm was regular. There was a grade I apical systolic murmur. Beyond these findings the examination was noncontributory.

Laboratory data: Blood Hinton negative. Urine, serial specimens: Specific gravity varied between 1.008 and 1.032, protein 4+ (18 grams in 24 hours), sugar negative, centrifuged sediment—red cells varied between 0 to 4 per high power field, white cells varied between 2 to 30 per high power field, many hyaline and granular casts per low power field. Blood: Red cells 5,200,000 per c.mm., hematocrit 48, sedimentation rate 32 mm. per hour, white count 8,600 with 77 per cent neutrophils and 3 per cent eosinophiles. Blood chemistry: Urea nitrogen 25 mgm. per cent, total protein 3.8 grams per cent, cholesterol 1,170 mgm. per cent, chlorides 108 m.eq. per liter, carbon dioxide combining power 24 m.M. per liter. Vital capacity

1,800 m.l. Venous pressure 90 mm. of saline. Circulation time (Decholin) 10 seconds. Congo red test negative. P.S.P. excretion: 15 minutes 10 per cent; total in 2 hours, 45 per cent.

X-ray examinations: Chest: Negative beyond clouding at right base and minimal fluid in the right costophrenic angle; heart normal in size and shape. Sinuses: Clouding of ethmoid cells and right frontal sinus, evidence of thickening in the sphenoid sinuses.

3. W. H. (M-65128), a 42-year-old male shipyard worker, was admitted to the Peter Bent Brigham Hospital on October 13, 1943, because of edema of 1 month's duration.

An anterior urethritis, in 1919, was followed by development of a stricture which was dilated, in 1927, with sounds. In 1942, an attack of prostatitis accompanied by frequency and nocturia was treated by his family doctor. The bacteriology of these infections is not known. In recent years, he had been subject to many sore throats.

His present illness began early in September, 1943, when, simultaneous with an acute upper respiratory infection characterized by chills, fever, and sore throat, puffiness was noted about his eyes. He had some soreness in the calves of his legs and in his feet. He subsequently developed ankle edema. Later, the edema became more generalized. He also had some malaise, headaches, and dizzy spells and had noted that his urine had been dark orange in color and decreased in amount. Ten days before admission he was put at bed rest on a low-salt, low-protein diet. During this time, he had had 2 episodes of paroxysmal nocturnal dyspnea.

Physical examination: Temperature 98.0° F., pulse 88, respirations 20, blood pressure 120/70 mm. Hg. A well-developed colored man exhibited marked puffiness about the eyes and moderate pitting edema of the ankles, sacrum and genitalia. Fundi showed no abnormalities. Teeth were carious and moderate pyorrhea was present. Although the throat was not red, the left tonsil was abnormally large and free of pus. Heart was normal. Lungs were clear. The prostate was not obviously enlarged. The remainder of the examination was non-contributory.

Laboratory data: Blood Hinton negative. Urine, serial specimens: Specific gravity varied between 1.005 and 1.014, protein 4+ (19 grams in 24 hours), sugar negative, centrifuged sediment—red cells varied between 1 and 8 per high power field, white cells varied between 3 and 15 per cent high power field, many hyaline and granular casts per low power field. Blood: Red cells, 3,400,000 per c.mm., hematocrit 32, sedimentation rate 15 mm. per hour, white cells 9,400 per c.mm., with 63 per cent neutrophils and 3 per cent eosinophiles. Blood chemistry: Nonprotein nitrogen 30 mgm. per cent, total protein 4.1 grams per cent, cholesterol 755 mgm. per cent, chlorides 113 m.eq. per liter, carbon dioxide combining power 27 m.M. per liter. Vital capacity 2,800 m.l. P.S.P. excretion—15 minutes 40 per cent, total 70 per cent in 2 hours. Multiple urine cultures showed staphylococcus aureus. Electrocardiograms: Low voltage, P-R interval 0.20 seconds, otherwise normal.

X-ray examinations: Lungs: Moderate pulmonary congestion. Heart: Twelve per cent above average by height-weight ratio. Sinuses: All sinuses showed thickening of the membranes but no fluid level. Teeth: Small areas of absorption along the roots of 2 molars.

4. D. S. (M-65112), a 30-year-old white male, was admitted to the Peter Bent Brigham Hospital on October 11, 1943, because of ankle edema of about 1 year's duration.

One and a half years before this admission, he was found to have marked albuminuria unassociated with other signs or symptoms. In the fall of 1942 he developed ankle edema. This gradually became more severe and spread to involve the entire body. He gained 15 pounds during the year preceding admission.

Physical examination: Temperature 98.4° F., pulse 84, respirations 20, blood pressure 140/90 mm. Hg. A well-developed and -nourished but pale middle-aged male showed pitting edema extending from his legs to his lower abdominal wall and involving his hands. The fundi were negative. The pharynx was normal. The lungs were clear. The heart was negative except for a soft systolic bruit at the apex. The remainder of the examination was noncontributory.

Laboratory data: Blood Hinton negative. Urine, serial specimens: Specific gravity varied from 1.009 to 1.018, protein 3+ (11 grams in 24 hours), sugar negative, centrifuged sediment—red cells varied between 0 to 4 per high power field, white cells varied between 0 to 5 per high power field, many granular casts and rare hyaline casts per low power field. Blood: Red cells 4,400,000 per c.mm., hematocrit 37, sedimentation rate 38 mm. per hour, white cells 7,000 per c.mm., with 62 per cent neutrophils and 3 per cent eosinophiles. Blood chemistry: Nonprotein nitrogen 27 mgm. per cent, total protein 4.8 grams per cent, cholesterol 1,000 mgm. per cent, chlorides 108 m.eq. per liter, carbon dioxide combining power 29 m.M. per liter. Vital capacity 4,200 m.l. P.S.P. excretion—15 minutes 20 per cent and a total of 75 per cent in 2 hours. Electrocardiogram normal.

X-ray examinations: Lungs: Clear. Heart: Five per cent below average size by height-weight ratio. Sinuses: Negative. Teeth: Negative.

5. K. N. (M-66677), a 40-year-old divorced stenographer, entered the Peter Bent Brigham Hospital on July 3, 1944, complaining of mild generalized edema of 5 weeks' duration.

At the age of 8, she had had an episode of generalized malaise and aching for which she was confined to bed for 2 weeks with the diagnosis of rheumatic fever, although no history of acute arthritis could be obtained, and there has never been any evidence of cardiac damage. In 1939, her urine was found to be entirely negative. For some 10 years, the patient had nocturia once a night and voided approximately 5 times during each day. For many years, the patient had suffered from recurrent attacks of tonsillitis, the last of which occurred in February, 1944, and lasted for 3 weeks. Patient had no other symptoms referable to the right maxillary sinus for a period of 2

weeks in 1942. No recrudescence of respiratory symptoms could be correlated with the present illness.

Physical examination: Temperature 99.0° F., pulse 84, respirations 20, blood pressure 130/80 mm. Hg. A well-developed and -nourished white female, without significant pallor or facial edema, presented minimal pitting edema of her ankles. Fundi were within normal limits. The pharynx was slightly injected. Both tonsils were present but not inflamed. There were numerous nontender, discrete, small cervical lymph nodes. Lungs were clear. The heart was normal. The remainder of the physical examination was negative except for a creamy white vaginal discharge.

Laboratory data: Blood Hinton negative. Urine, serial specimens: Specific gravity 1.010, protein 3+ (7 gm. in 24 hours), sugar negative, centrifuged sediment—red cells varied between 1 to 12 per high power field, white cells varied between 0 to 5 per high power field, occasional hyaline and fine granular casts. Blood: Hemoglobin 12.5 gm. per cent, hematocrit 39, sedimentation rate 45 mm. per hour, white cells 9,500 per c.mm., with 58 per cent neutrophils and 3 per cent eosinophiles. Blood chemistry: Urea nitrogen 9 mgm. per cent, total protein 5.6 gm. per cent, cholesterol 430 mgm. per cent. Throat cultures showed normal throat flora. Serial urine cultures were sterile. Culture of tooth root following extraction showed alpha streptococci and staphylococcus albus. P.S.P. excretion—15 minutes 45 per cent and a total of 75 per cent in 2 hours. Congo red test negative. Basal metabolic rate +3 per cent. Vital capacity 3,600 m.l. Electrocardiogram normal.

X-ray examinations: Chest: Clear. Heart: Six per cent below average in size by height-weight ratio. Sinuses: Negative. Teeth: Areas of absorption around the roots of the right lower first molar. Retrograde pyelograms: Negative.

6. E. B. (M-67041), a 44-year-old white housewife, was readmitted to the Peter Bent Brigham Hospital on September 11, 1944, because of increasing lassitude, exertional dyspnea, and generalized edema over the past 3 months.

In the fall of 1943, she was found to have hypertension with blood pressures as high as 160/110 mm. Hg and moderate proteinuria. She felt relatively well until a few months prior to the present admission at which time she began to complain of loss of vigor, mild exertional dyspnea, slight generalized edema, and lack of mental acuity.

Physical examination: Temperature 98.4° F., pulse 82, respirations 20, blood pressure 175/105 mm. Hg. A well-developed and well-nourished middle-aged woman, clear in mind, showed a slightly pale skin and generalized puffiness which graded in the arms, sacrum and legs into mild but pitting edema. Beyond abnormally narrow retinal arterioles, examination of the fundi was negative. The pharynx showed no evidence of infection. The lungs were clear except for an occasional crackling rale at the right base. Beyond soft nontransmitted apical and basal systolic murmurs, no further cardiac abnormalities were found. The remainder of the physical examination was noncontributory.

Laboratory data: Urine, serial specimens: Specific gravity varied between 1.010 and 1.026, protein 4+ (5 grams in 24 hours), sugar—initially 4+, with regulation of diabetes 0, centrifuged sediment—red cells varied between 2 to 4 per high power field, white cells varied between 0 to 1 per high power field, occasional hyaline and granular casts. Blood: Red cells 4,000,000 per c.mm., hematocrit 40, sedimentation rate 50 mm. per hour, white cells 10,300 per c.mm. with 73 per cent neutrophils and 4 per cent eosinophiles. Blood chemistry: Urea nitrogen 20 mgm. per cent, total protein 4.1 grams per cent, cholesterol 880 mgm. per cent, chlorides 104 m.eq. per liter. Basal metabolic rate -21. Bromsulphalein test—2 per cent retention after 40 minutes. Venous pressure 115 mm. saline. Circulation time (Decholin) 28 seconds. Electrocardiogram, low voltage, with occasional premature ventricular beats.

X-ray examinations: Lungs: Clear. Heart: Twenty per cent above average size by height-weight ratio.

7. R. S. (M-65187), a 29-year-old white married welder, was admitted to the Peter Bent Brigham Hospital on October 25, 1943, with the complaint of "kidney disease" of 6 months' duration.

Patient had had a chronic cough with numerous exacerbations in the winters during the previous 2 decades. Clubbing of the fingers had been present for many years.

In May, 1943, following a period of generalized malaise, he developed swelling of his ankles and was found to have both albuminuria and marked microscopic hematuria. He was treated at home by his local physician with bed rest, low-protein and low-salt diet. There had been no previous acute infection. His edema gradually subsided, but he developed hypertension with levels as high as 170 mm. Hg systolic. Albumin, red and white cells persisted in the urine. He returned to work, however, and did well until October, 1943, when he again developed edema of his legs, and he noted that his urine was scant and smoky. He went to bed 2 weeks before admission and his ankle edema disappeared. There had been intermittent nocturia during the 8 months preceding admission, but there had been no other symptoms suggesting genito-urinary disease. His only cardiovascular manifestation was minimal exertional dyspnea developing since his first acute episode.

Physical examination: Temperature 97.8° F., pulse 78, respirations 18, blood pressure 150/90 mm. Hg. A well-developed and nourished man in early middle age, presented a sallow complexion. Although his face was not puffy, there was minimal pitting edema of both ankles and sacrum. Fundi were within normal limits. Although the patient's tonsils were present, neither they nor the pharynx appeared infected. The trachea was deviated slightly to the left. The lungs were negative. To percussion the heart appeared moderately enlarged to the left, but beyond a soft apical systolic murmur no other abnormalities were found. Prostate was normal. The remainder of the examination was noncontributory.

Laboratory data: Blood Hinton negative. Urine, serial specimens: Specific gravity varied between 1.006 and 1.012, protein 3+ (12 grams in 24 hours), sugar negative, centrifuged sediment—red cells varied between 1 to

2 per high power field to a sediment loaded with red cells such that other elements were obscured, white cells usually between 3 to 10 per high power field, on 2 occasions many more white cells were seen among large quantities of red cells, casts varied between 1 and 20 per low power field. Blood: Red cells 4,400,000 per c.mm., hematocrit 30, sedimentation rate 61 mm. per hour, white cells 10,000 per c.mm., with 70 per cent neutrophils. Blood chemistry: Urea nitrogen 46 mgm. per cent, total protein 5.6 grams per cent, cholesterol 340 mgm. per cent, chlorides 116 m.eq. per liter, carbon dioxide combining power 20 m.M. per liter. Vital capacity 4,500 ml. Overnight urine concentration test—maximum specific gravity 1.010. Pituitrin concentration test—maximum specific gravity 1.008. P.S.P. excretion—15 minutes 10 per cent, and a total of 65 per cent in 2 hours. Electrocardiogram normal. Serial urine cultures showed either no growth or an occasional staphylococcus albus or alpha streptococcus.

X-ray examinations: Chest: Clear. Heart: Slightly enlarged to right and left. Sinuses: Negative beyond thickened membranes in left antrum. Lipiodol examination of bronchi: No obvious bronchiectasis although bronchus to right lower lobe filled poorly. Teeth: Negative.

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THE TOXIC FACTORS IN EXPERIMENTAL TRAUMATIC SHOCK.

I. PHYSIOLOGIC EFFECTS OF MUSCLE LIGATION IN THE DOG¹

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The experimental study of traumatic shock requires the use of a reproducible procedure for damaging muscles and surrounding tissues. Since the classical method of Cannon and Bayliss (1), many techniques have been employed (2 to 5). By the use of such methods, a relatively uniform clinical result can be obtained in a series of experiments, but the exact amount of tissue injured and of accumulated fluid cannot be measured accurately. Collection of the traumatic exudate is likewise a difficult procedure although its composition has been the subject of investigation (6).

In the course of making a study of electrolyte and water shifts in shock, it became desirable to utilize a method whereby a known or measurable amount of tissue could be damaged by temporary interruption of its blood supply. The method described in this paper was evolved in order to accomplish this.

This method also has certain other important advantages which have made it very useful in the study of the etiology and therapeutics of traumatic shock. It is traumatically less severe than many methods, and therefore does not usually produce overwhelming shock. This provides the opportunity of differentiating possible early and primary changes in physiological responses which might be obscured with more vigorous techniques. Moreover, it is suitable for the study of the effects of therapeutic procedures to which an excessively traumatized animal could not be expected to respond.

this accumulation is largely limited to the lower part of the legs, it is possible to estimate it as a function of time by measuring volume changes in the limbs up to the groin. The procedure has also been modified so that the accumulating fluid can be collected completely; in this way, factors affecting the rate of extravasation in a traumatized area can be evaluated, and the fluid itself can be obtained in large quantities for investigation of its chemical composition, its physiologic properties, and its bacterial flora.

TECHNIQUE OF OPERATION

Aseptic precautions are used throughout. All animals were anaesthetized by means of sodium pentobarbital, 30 mgm. per kgm. given intravenously. The hair of both hind limbs of the dog is carefully clipped and shaved from the paw to the inguinal fold. The shaved skin is then scrubbed thoroughly with soap and water, dried with ether, and painted with tincture of iodine. The hips are then acutely flexed, the legs brought into extension and anchored to the table. The legs are draped with sterile towels and a small laparotomy sheet. A linear incision is made in the middle of the skin of the calf extending from the level of the attachment of the tendo Achillis to a point just proximal to the popliteal space. The skin edges are protected by sterile towels or gauze sponges which are secured by a continuous silk suture. The fascia overlying the calf muscles is then incised over the entire length of the wound, and retracted laterally. By blunt dissection with the finger, the triceps surae muscles (gastrocnemius and soleus) are isolated together from the plantaris muscle, the posterior tibial and peroneal nerves, and the muscles immediately posterior to the tibia and fibula. This frees the triceps surae muscles except at their origin

When the desired position for the rubber tourniquet is obtained, the loop is closed as tightly as possible and secured by a tape tie which was previously placed (see diagram, Figure 1). The tendo Achillis is ligated so as to occlude the blood flow at that end. The incision is closed.

At the end of 5 hours, the wound is prepared, draped, and opened at its proximal end. The tourniquet is divided and removed. The arteries distal to the site of the tourniquet are palpated, and the veins are emptied by mild manual pressure and allowed to refill in order to estimate the integrity of the circulation. Other methods to estimate

this are described later. The wound is then resutured and the limbs returned to the extended position.

When complete isolation of the triceps surae from the surrounding tissues is desired, the technique is modified at the time of the first procedure as follows. The tendo Achillis is divided close to its insertion and the cut ends ligated. A rolled rubber sheath is then unrolled over the muscles as far as the tourniquet. The proximal (open) end is anchored in position by a small rubber band. The skin and fascia are then closed in one layer by a continuous silk suture and the limbs are brought into extension. Five hours later the tourniquet is removed as described

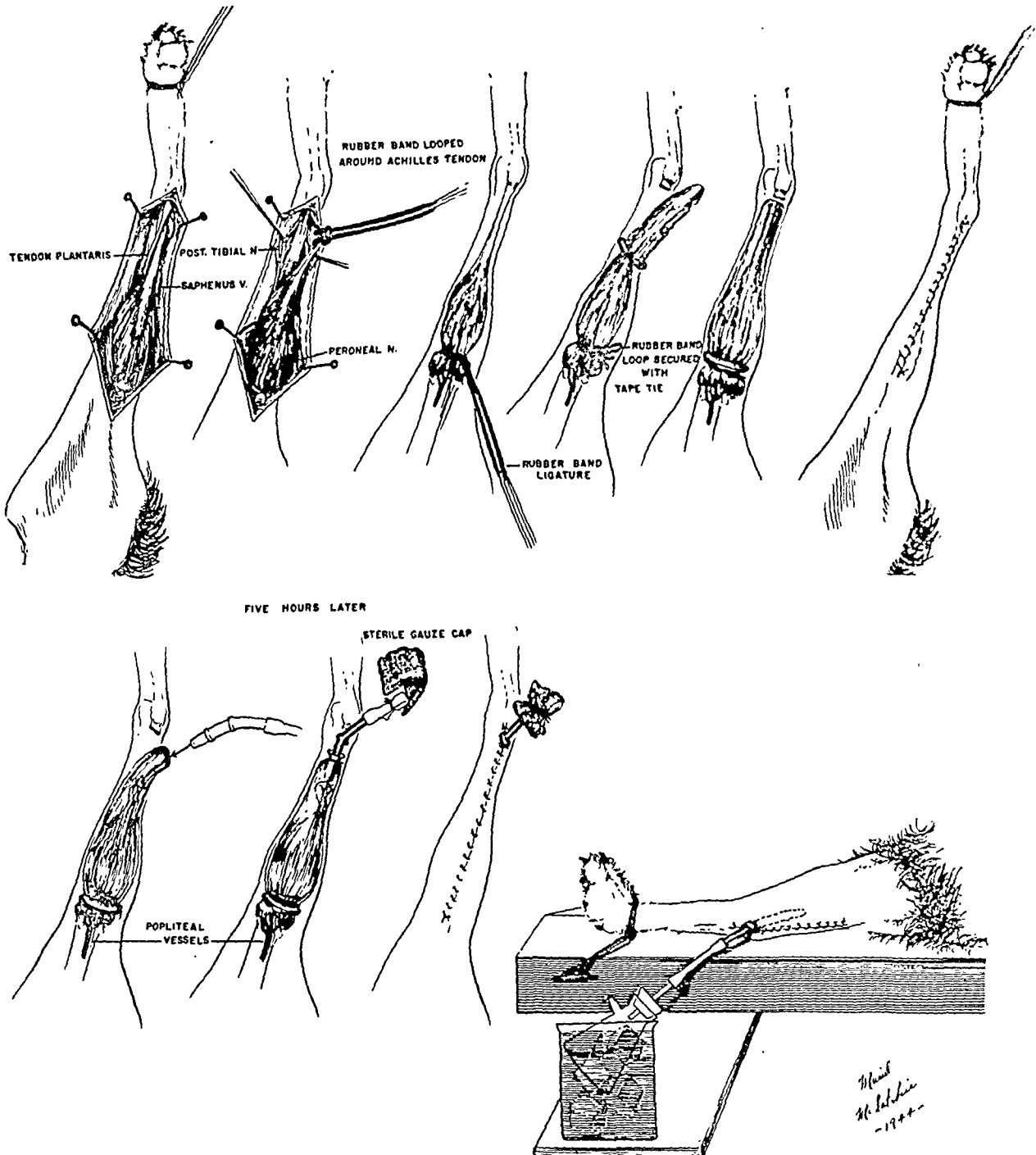


FIG. 1. DIAGRAMS ILLUSTRATING TECHNIQUE OF MUSCLE LIGATION (For explanation see text)

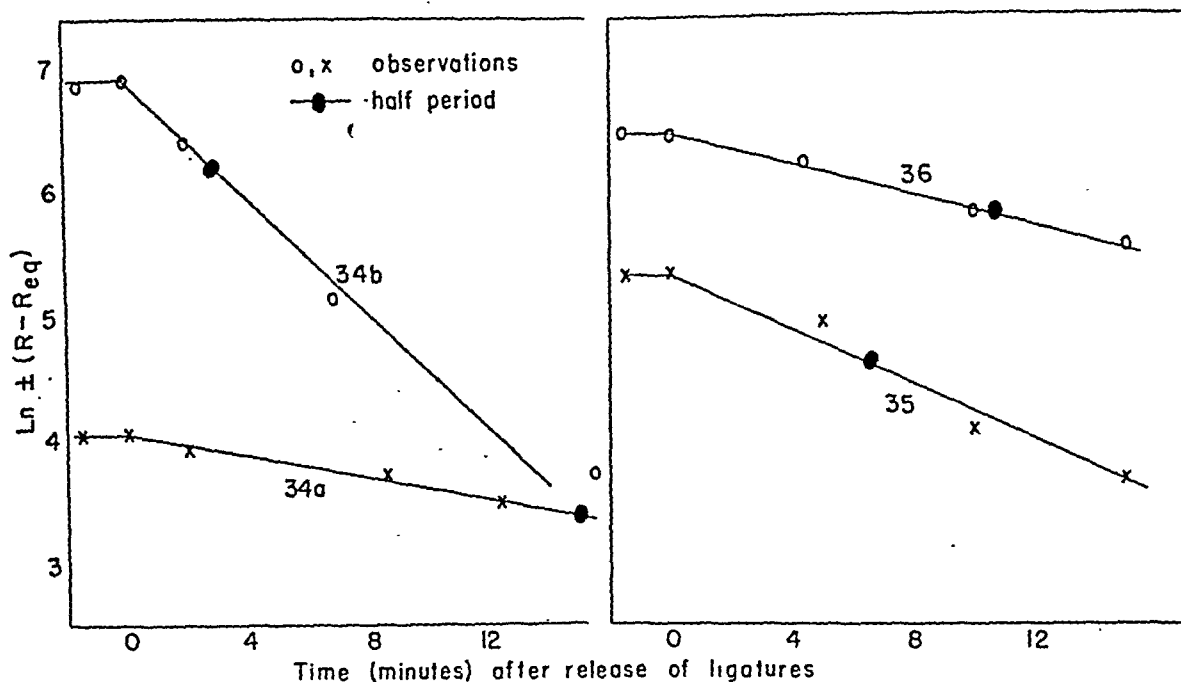


FIG. 2. GRAPH OF CHANGES IN MEASURED RADIOACTIVITY FOLLOWING RELEASE OF LIGATURES, AFTER INJECTING Na^{24} INTO LIGATED MUSCLES

The natural logarithm of the (positive) difference between each reading and the equilibrium reading* has been plotted against time. The half-period in each case is indicated on the curve. Note that each set of observations, plotted in this way, approximates a straight line, indicating that equilibrium is approached through a simple exponential curve. As noted in the text, the curves thus calculated always slope downwards whether loss or uptake of the isotope is measured.

34a. Readings taken in left axilla after release of right muscle group (containing Na^{24}).

34b. Readings from area of left muscle group following its subsequent release.

35. Readings taken over right muscle group (containing Na^{24}) following its release.

36. Readings from left axilla, as in 34a.

* All readings corrected for background and for decay of the isotope.

above and the wound closed. If fluid collection from the tourniqueted muscle is desired, the following procedure is utilized.

Fluid collection. The wound is reopened throughout its entire length and the rubber tourniquet is divided and removed. The open end of the sheath is then carried as close as possible to the muscle origins. The closed distal tips of the sheath is incised and the end of a curved glass tube about 1 cm. in diameter is inserted. This is secured by several ligatures placed about the sheath above and below a collar on the tube. At the distal end of the tube is placed a length of rubber tubing, which had been previously folded on itself, secured by a ligature, and covered by a sterile gauze sponge (see Figure 1). The tube is then led out of the distal end of the incision or through a stab wound on the lateral skin flap. The skin and fascia are closed in one layer with a continuous silk suture and the glass tube is anchored to the skin by a purse-string suture which is tied proximal to a collar on the tube. The legs are brought into extension and secured. The gauze cap is removed from the rubber tubing and the tubing unfolded. The distal end of the tubing is then

placed over a sterile glass tube which had been inserted in a rubber stopper attached to a sterile suction flask. Sterile cotton is inserted into the open end of the side arm of the flask. The flask is then immersed in a beaker of ice and water which is placed on a shelf attached to the operating table. Thus the fluid which exudes from the muscle is collected in the flask. Fluid comes out rapidly, usually after a short lag period, and after some hours the flow subsides. The collection is continued for about 5 hours or until the muscles cease to yield fluid.

Reestablishment of circulation following anoxia. In addition to observations on arterial pulsation in muscles following release of ligatures, radioactive tracers were employed in some cases to determine reestablishment of circulation. Just before the ligatures were released, 1 or 2 ml. of isotonic sodium chloride containing a suitable amount of radioactive sodium (Na^{24}) were injected into the substance of one of the tied muscles. Distribution of the labelled sodium chloride with that outside the muscle was followed by means of readings taken on a Geiger-Müller counter held in position over the injected muscle, the uninjected muscle, or the left axilla. Following re-

lease of tourniquet, there was a prompt decrease in radioactivity over the injected area and increase over other areas in all cases. Final readings were taken after equilibrium had apparently been reached.

On the supposition that redistribution of the isotope inside and outside the muscle might reach equilibrium through a simple exponential curve, the logarithms of the differences between each reading and the final equilibrium reading were plotted as ordinates against the time after release of ligatures. It will be noted that by plotting the logarithm of each difference as a positive value, all curves will show a downward slope whether one is measuring loss of isotope from the injected area or its increase in other areas ($y = K(1 - \Sigma^{-kt})$ or $y = K\Sigma^{-kt}$, respectively). The resulting graphs showed approximately straight lines in all cases (Figure 2), and from these slopes half-periods have been calculated (i.e., time required for the exchange to become half completed). A tabulation of the findings follows:

Dog 34. Muscles were isolated by sheaths (not drained). Na^{24} was injected into the right gastrocnemius muscle. Readings taken over the left axilla (a) showed increase of radioactivity with half-period 15 minutes, following release of right muscle group. Thirty minutes later, the left muscle group was released while readings were made over this area (b), indicating penetration of Na^{24} into the uninjected muscle. Half-period $3\frac{1}{2}$ minutes.

Dog 35. No sheaths used in this experiment. Na^{24} injected into right gastrocnemius muscle; axillary readings made while ligature was released. Half-period $10\frac{1}{2}$ minutes.

Dog 36. Muscles were isolated by sheaths (not drained). Na^{24} injected into right gastrocnemius muscle;

axillary readings made while ligature was released. Half-period $10\frac{1}{2}$ minutes.

In 2 additional experiments the state of the muscle circulation was tested before and after release of ligatures by a semi-quantitative procedure. Immediately following application of ligatures, 1 ml. of 1 per cent phenol red (10 mgm.) was injected into the substance of each muscle group. Urinary excretion of the dye was then followed and calculated as percentage of the injected amount. The results follow:

Dog 47. Muscles were sheathed and drained. Excretion of the dye in the first hour after application of ligatures was 3.8 per cent. Excretion in following 4 hours, 1.5 per cent. In 35 minutes after first release 4.6 per cent was excreted; second ligature was then released and 5.9 per cent was excreted in the ensuing 40 minutes. Total excretion in 14 hours, 34.4 per cent.

Dog 48. Sheaths were not used. During 5-hour period of application of ligatures, 0.4 per cent of the dye was excreted. In 30 minutes after first release, 5.7 per cent; in 1 hour after second release, 11.2 per cent. Total in 10 hours, 33.9 per cent.

In the first case there was escape of dye soon after injection, which did not occur in the second case. In both cases a prompt excretion occurred following release of each tie, confirming the results with Na^{24} .

The above results show that the procedure usually causes a nearly complete cessation of circulation in the muscles, and that prompt reestablishment of the circulation after release of ligatures is the rule.

Mass of muscle involved. In a few cases the triceps surae groups were weighed either before or after release of ligatures. Three specimens taken while ties were in

TABLE I

Results of muscle tying procedure

(Average values for blood pressure, venous oxygen content and blood hemoglobin at various stages in the procedure)

	Number of dogs	Initial	Before release of ties	30 minutes after release of ties	6 hours after release of ties	(Final) range
Without drainage—no shock						
Blood pressure	7	113	130	124	100	90 to 110
mm. Hg						
Venous O_2	2	14.3	14.1	12.5	9.1	8.4 to 9.8
volumes per cent						
Hemoglobin	2	12.8	16.6	16.6	17.8	16.0 to 19.6
grams per cent						
Without drainage—shock*						
Blood pressure	3	133	141	125	59	46 to 68
mm. Hg						
With drainage—no shock						
Blood pressure	35	128	146	134	118	80 to 140
mm. Hg						
Venous O_2	8	13.3	14.3	11.2	12.2	6.0 to 16.7
volumes per cent						
Hemoglobin	7	13.0	13.5	13.8	16.3	14.8 to 20.8
grams per cent						

Note: The 10 dogs submitted to simple muscle tying without drainage have been divided into 2 groups depending upon whether shock occurred (defined arbitrarily as blood pressure below 70 mm. Hg). No dogs in the group treated with drainage showed signs of shock.

* No values available for venous oxygen and hemoglobin.

place had a mean weight equivalent to 0.35 ± 0.08 per cent of body weight; 7 specimens taken 6 hours after release weighed 0.52 ± 0.08 per cent of body weight. Thus, the total mass of tissue involved in the bilateral procedure includes roughly 1 per cent of body weight.

RESULTS

Uncomplicated muscle ligation. Ten dogs were subjected to the procedure without drainage of fluid, the muscles being tied bilaterally for a $5\frac{1}{2}$ -hour period. Six hours after release of the ligatures, 3 of the 10 dogs had blood pressures below 70 and were considered to be in shock. Two of these died within the next hour and the third was sacrificed. The remaining 7 had blood pressures ranging between 90 and 110, and were considered not to be in shock. Two of these were sacrificed within 7 hours and the remainder were allowed to survive from 20 to 38 hours. In Table I, we have summarized observations on these dogs which have been divided into 2 groups depending upon whether or not shock occurred. In 2 dogs without shock, observations were made on venous oxygen content and on hemoglobin. A moderate hemoconcentration was observed, and the venous oxygen levels declined somewhat suggesting a decrease in cardiac output.

Procedure with drainage. All 35 animals in this group failed to show evidence of shock within 6 hours after release of ligatures. Table I shows that there was little change in the venous oxygen content, but hemoconcentration regularly occurred after release.

In addition to the experiments tabulated here, a number of dogs were subjected to considerable bleeding (over 0.5 per cent of the body weight equivalent in blood), and these will be discussed elsewhere. Seven dogs done without sterile precautions, of which 5 received 500 mgm. sodium thiocyanate intravenously, and all of which were bled an amount exceeding 1 per cent of body weight, have been omitted from the present series. Although all of these dogs developed shock within 5 hours after release, it is clear that the procedure was complicated by other factors potentially detrimental to the circulation.

Accumulation of fluid. Local swelling was almost invariably observed following release of ties. It will be seen (Table I) that hemoconcentration occurred in those animals where drainage was not employed, as well as in those whose extrava-

sated fluid was drained. Unfortunately, there are no data to show the degree of hemoconcentration in the 3 which developed shock.

In an attempt to improve the yield of fluid from drained muscles, slow infusions of physiologic salt solution were given intravenously over a 2 to 3-hour period beginning before release of the ligatures. The amount of fluid given was approximately equal to the animal's theoretical plasma volume. Such experiments are compared with controls in Table II. It is seen that there is wide variation in fluid output in both groups of animals

TABLE II

Effect of intravenous saline infusion on production of fluid following muscle ligation

	Number of dogs	Fluid produced ml. per kgm. median value	Range of values ml. per kgm.
No infusion	23	5.3	0 to 20
Saline infusion*	38	15.0	2 to 33

* Average amount of fluid administered = 55 ml. physiological saline per kgm. body weight.

but that the average output is approximately trebled when the saline transfusion is given. Nearly one-fifth of the administered fluid was recovered by muscle drainage.

Absorption of intracellular constituents. In 4 dogs, 2 treated with drainage, and 2 without, plasma creatine determinations were made (7). In Figure 3, it is seen that the plasma creatine, which is essentially normal during the period of

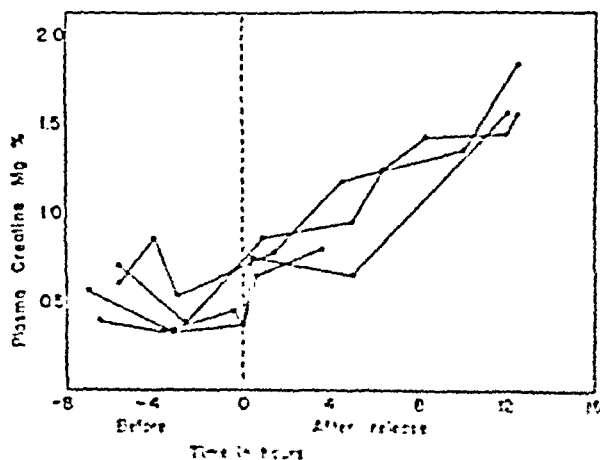


FIG. 3. GRAPH ILLUSTRATING CHANGE IN PLASMA CREATINE CONCENTRATION AFTER RELEASE OF MUSCLE LIGATURES

ligation, rises steadily for at least 12 hours after release. This confirms the observation (8) that whole blood creatine rises following muscle trauma. That muscle injured by crushing loses 65 to 70 per cent of its creatine has been shown (9). It is interesting that none of the 4 dogs on which determinations were made developed shock.

The steady increase in plasma creatine in dogs not in shock is especially interesting since it suggests that absorption of a component of muscle occurs in the blood stream. Plasma potassium and phosphate values, on the other hand, have remained essentially constant until shock is present and rise only after renal function has essentially ceased.

Bacterial contamination. Nearly all of the fluids obtained have shown bacterial contamination, at times very heavy, in spite of the aseptic techniques employed. The possible relation of this contamination to the shock produced has been discussed in a preliminary paper (10) and is considered more fully in another paper of this series (11).

DISCUSSION

The procedure described here was devised to produce a controlled type of traumatic damage (simple ischemia), over a specified time interval, to a definite amount of muscle. Despite the small amount of muscle concerned, shock has occurred in some instances even when other procedures, such as moderate blood losses, were not superimposed. It is noteworthy that local fluid loss and absorption of tissue constituents into the circulation have both been shown to accompany the procedure.

By means of an accurate technique for collecting the fluid which exudes from the anoxic muscle, a separation is possible between those factors present in this fluid and the possible ones absorbed directly into the blood stream, as exemplified by creatine. The absence of shock where extracellular fluid is drained implies that such toxins

as may escape directly into the circulation are inadequate in themselves to produce shock when a small muscle mass is involved.

It is surprising how much fluid exudes from these small muscle bundles after anoxia is stopped. It appears obvious that solutes must be reabsorbed into the general circulation when the fluid is allowed continued contact with surrounding tissues. This, of course, would increase the absorption of any toxin which might be present.

There are advantages in not traumatizing an excessive amount of muscle tissue. Certain studies involving differential factors in the production and therapy of traumatic shock may be made when a minimal trauma is used, and yet may be obscured by the overwhelming shock which follows excessive muscular involvement.

Experiments bearing on these questions will be described in the following papers of this series.

SUMMARY

A method is described for the production of ischemic damage in a reproducible way to a known amount of muscle with subsequent reestablishment of circulation. The amount of tissue involved comprised about 1 per cent of body weight.

This method occasionally produces shock (3 out of 10 cases) when uncombined with other procedures detrimental to the circulation.

Both local exudation of fluid and absorption of muscle constituents into the blood stream follow the removal of tourniquets. It is possible, therefore, to assay the relative rôles of these 2 factors in the production of shock by a reproducible traumatic procedure.

Quantitative collection of muscle exudate can be accomplished by this technique.

Intravenous saline transfusions increase the amount of fluid exudation about traumatized tissue.

Advantages of a quantitative method of this type in the critical study of traumatic shock are discussed.

Bibliography follows Paper VI of this series.

THE TOXIC FACTORS IN EXPERIMENTAL TRAUMATIC SHOCK.

II. STUDIES ON ELECTROLYTE AND WATER BALANCE IN SHOCK¹

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It is recognized that capillary permeability may be altered locally in burned or traumatized areas, and that the consequent loss of fluid from the blood stream is important in producing shock. It is less certain whether general changes in capillary permeability exist outside of injured areas, except as a very late phenomenon. It is desirable to know also whether changes in permeability of tissue cells occur in shock, locally or generally, along with the reduced capillary blood flow and resulting anoxia. The present study was made in order to determine whether, in various forms of shock, there are important shifts of water or electrolytes between cells and extracellular spaces.

A study was made of the body water distribution in 5 dogs in which shock was produced by hemorrhage and in 3 dogs by hammer trauma to a hind leg (12). It was concluded that there is a passage of water from extracellular spaces into cells following hemorrhage, while in traumatic shock there is an unexplained passage of water out of cells. Two workers (13) studied the water, potassium, and phosphorus content of tissues in 6 control dogs and 4 dogs in which shock occurred following light blows on the thigh, and in a larger series of rats that went into shock following rotation in a drum (5). Tissue analyses made at the verge of death revealed increases in muscle potassium and phosphorus of dogs in shock, whereas in rats the liver lost potassium and phosphorus. The increases found in dogs were considered to result from loss of potassium from traumatized areas. A marked decrease in the potassium content and an increase (double or

higher) in the chloride content of traumatized muscles was reported with a slight decrease in plasma chloride, no consistent change in plasma sodium, and a slight rise in plasma potassium, becoming marked just before death (6). In crush injury it has been observed (9) that the necrotic muscle may lose 50 to 90 per cent of its potassium and 65 to 70 per cent of its creatine.

METHODS

Shock was produced in dogs with the following techniques:

(a) Hemorrhage (6 dogs). This was done by serial bleedings until the blood pressure was below 60, after which continuous observations were made until death. The initial bleeding was made equal to 2 per cent of the body weight; subsequent smaller bleedings were done at intervals of $\frac{1}{2}$ to 1 hour.

(b) Application of a Duncan-Blalock press (6) (5 dogs) to one thigh with a pressure of 500 pounds over a period of 5 hours, following which the press was removed.

(c) Application of a tourniquet as high as possible on one hind leg with sufficient pressure to obliterate femoral artery pulsation. The tourniquet was removed after 5 hours (2 dogs).

(d) The muscle ligation procedure which has been described (7) (7 dogs). In these animals the triceps surae muscle groups were rendered ischemic by tying for 5 hours, after which the ties were removed.

All of these procedures were carried out under sodium pentobarbital anesthesia. Animals were originally given intravenously 30 mgm. sodium pentobarbital per kgm. with additional doses of 30 to 60 mgm. only when restless. In addition, one control dog was maintained under sodium pentobarbital anesthesia and subjected to other procedures common to the experiments over a 10-hour period and was then sacrificed.

Most of these dogs were given intravenous injections of radioactive sodium (Na^{24}) or of sodium thiocyanate (250 to 500 mgm.) or both and serial blood samples taken for determination of extracellular space throughout the experiment; at the same time hematocrits, hemoglobin and plasma sodium (15), potassium (16), and chloride (17) determinations were made. Thiocyanate determinations

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The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts General Hospital.

tions were made by the method of Chesley (18). Total blood removal for these determinations ranged between 100 and 150 ml. Early in the experiments, 3 to 4 grams of rectus muscle and liver tissue were removed from most of the dogs by sterile operative techniques, for control tissue analyses. At death, rectus muscle and liver were removed, and in experiments dealing with trauma or ischemia samples of sartorius and gastrocnemius muscles were also taken. Thus in the Duncan-Blalock press group, one sartorius muscle was taken from the crushed area, and a gastrocnemius muscle was removed from the area rendered ischemic below the clamp in addition to the uninjured muscles from the control legs; in the muscle-tying group, samples of an uninjured sartorius and a gastrocnemius muscle which had been rendered ischemic were obtained. These tissues were divided into 3 samples of 1 to 2 grams each, weighed, and kept in petri dishes frozen over CO₂ snow. Analyses were made for water, fat, hemoglobin, sodium, potassium, nitrogen, and radio-sodium. With certain slight modifications, the handling and analysis of tissues were done according to the procedures described by Lowry and Hastings (16). Tissue sodium determinations were done by the method of Leva (15), and hemoglobin by the procedure described by Cohn (19). Tissue chloride determinations became contaminated by hydrochloric acid fumes in the laboratory hood and were therefore discarded. From the results of blood and tissue analyses, sodium space was determined in the tissues, and cell potassium was calculated in terms of milliequivalents per kilogram of wet cells, as if sodium space represented true extracellular volume. The methods of calculation were similar to those of Hastings and Eichelberger (20) except that cell potassium has been expressed in terms of intracellular volume² instead of intracellular water. In many animals, one or more lobes of the liver were perfused *in situ* by allowing isotonic sucrose solution to flow through the portal vein at a pressure of 100 to 200 mm. of solution. This was continued until blood was practically removed, and analyses were done on tissues and perfusates.

RESULTS

Total extracellular space. The results of determinations of thiocyanate and radioactive sodium spaces are summarized in the first table. In all cases, the Na²⁴ and thiocyanate lost by removal of blood were deducted from each succeeding calculation, so that the resulting values indicate extracellular space remaining in the animal.

All of the variations seen are practically within the limits of error of the analytical methods. It will be seen that there is a decrease in thiocyanate space amounting to 2.3 per cent of body weight in hemorrhaged dogs. The individual dogs showed changes ranging between 6.6 per cent loss

² Strictly speaking, non-sodium volume.

TABLE I
Extracellular space in shock, by thiocyanate and radioactive sodium
Expressed as percentage of body weight. Mean values for each type of experiment.

Hemor- rhage	Control deter- mination	1 hour after hem- orrhage	1 hour before death	Terminal value	Number of dogs
Na ²⁴	32.4	32.8	33.3	32.7	4
SCN	33.2	31.2	30.5	30.9	5

Duncan- Blalock press	Control deter- mination	Before removal of clamp or tie	1 hour after removal	2 hours after removal	Ter- minal value	Num- ber of dogs
Na ²⁴	32.1	32.7	32.1	35.0	31.8	1
SCN	29.8	31.3	31.9	32.5	32.6	4
Tourniquet						
SCN	36.0	38.0	40.7	43.3	42.0	1
Muscle ligation						
Na ²⁴	30.1	30.5	31.0	32.4	33.5	2
SCN	37.7	37.0	37.2	36.7	35.4	4

and 1.8 per cent gain. Since the mean loss is of the same order of magnitude as the amount of plasma removed in bleeding, this shift probably does not represent a shift of water from extracellular space to cells, but serves to indicate that no such shift occurred. There was a mean increase of extracellular space in the Duncan-Blalock press group of plus 2.8 per cent; it appears that this shift is mainly due to diffusion into the extracellular space of the leg after removal of the press, since the substances were injected after applying the press. This will be discussed in the next sections.

In agreement with the interpretation that there is little or no shift of water between cells and the extracellular compartment, it will be seen from Table II that plasma chloride values do not show any considerable change during the course of the experiments. Potassium rises considerably and, as has been shown (6), this rise occurs mainly just before death. The averages suggest a terminal rise in plasma sodium in dogs submitted to hemorrhage, but the individual data show a decrease in 1, increase in 3, and no change in 2.

Tissue analyses. Two sets of tissue analyses were obtained for sodium and potassium. Values obtained by the 2 sets of analyses differed slightly, and in the case of each electrolyte 1 set was consistently higher than the other. The values shown

TABLE II

Mean values for plasma chloride, sodium and potassium in shock

	Plasma Cl m.eq./l.	Plasma Na m.eq./l.	Plasma K m.eq./l.
Hemorrhage (6 dogs)			
Before hemorrhage	112	139	3.61
1 hour after	113	141	3.51
2 hours after	113	139	3.61
3 to 4 hours after	114	140	4.10
Terminal	116	149	5.90
Duncan-Blalock press (5 dogs)			
Control	110	135	3.38
Clamp on 1 hour	110	141	3.15
Before removal	108	143	4.00
1 hour after removal	108	141	4.15
Terminal	107	137	6.82
Muscle ligation (7 dogs)			
Control	114	142	3.84
1 hour after tying	112	144	4.27
Before release	113	149	4.03
1 hour after release	113	148	4.03
Terminal	113	144	6.41

in Table III are from the set of analyses in which control tissues gave closest agreement with published tissue analyses (20, 21). In general, the trends observed were followed in both pairs of analyses and in the case of sodium; the same

trends were also seen in radioactive sodium analyses.

As shown in the table, significant changes in tissue composition are seen only in the case of analyses of crushed sartorius muscle in the Duncan-Blalock press group and the tied triceps surae muscle. In these muscles, the sodium space is increased and the potassium content is decreased correspondingly. It is of interest that the muscles which were ischemic because of their position below the Duncan-Blalock press do not show these changes, although muscles made ischemic by the ligation technique are similar to crushed muscles, suggesting that operative isolation and handling of the muscles in the ligation procedure may be partly responsible for the changes, or, more likely, that collateral circulation occurred through bone in experiments using the press.

It appears that the mean sodium space of rectus muscles after hemorrhage is somewhat less than that of muscles removed at biopsy. This can partly be explained by lower blood content determined from hemoglobin analyses (3.5 per cent as against 7.4 per cent). After correcting for this, the significance of the difference is doubtful.

Results of liver perfusion at death showed that under the conditions used, blood was virtually re-

TABLE III

Sodium space of tissues, tissue potassium, and cell potassium concentrations*

	E_{Na}			K_t			K_c			K/N		
	Mean	σ	n	Mean	σ	n	Mean	σ	n	Mean	σ	n
Muscle												
Biopsy	28.9	7.4	9	81.7	5.8	14	116.3	10.4	9	2.58	0.19	6
Hemorrhage	18.3	6.9	4	100.6	10.8	4	124.8	21.3	4			0
Duncan-Blalock press												
Normal	29.7	6.6	12	82.0	14.0	13	115.4	20.6	12	2.51	0.29	13
Crushed	59.5	10.1	4	38.8	12.7	4	89.7	11.2	4	1.51	0.47	4
Ischemic	27.6	1.6	4	80.5	20.1	4	102.6	25.7	4	2.56	0.26	4
Muscle ligation												
Normal	32.6	3.0	4	85.1	5.5	4	123.0	5.8	4	2.77	0.19	4
Ischemic	60.8		2	52.0		2	121.0		2	1.85		2
Liver												
Biopsy	34.4	5.9	10	85.9	13.1	12	117.9	13.9	9	2.40	0.28	8
Hemorrhage	26.7		2	86.0		2	116.5		2			0
Duncan-Blalock press	36.4	2.9	5	79.0	12.7	4	126.0	20.0	4	2.26	0.23	4
Muscle ligation	33.7		2	73.2		2	108		2	2.19		2

σ = standard deviation.

n = number of tissues analyzed.

E_{Na} = sodium space in ml. per 100 grams of tissue.

K_t = tissue potassium, m.eq. per 100 g. wet tissue.

K_c = intracellular concentration of potassium, m.eq. per 100 g. wet cells.

K/N = ratio of potassium (m.eq.) per nitrogen (grams) in tissue.

* Calculations are based on fat-free tissue weights.

moved from the organs after perfusion with about 4 volumes of isotonic sucrose solution. At this time, extracellular ions (sodium, thiocyanate, chloride) and also considerable amounts of potassium were being removed in decreasing amounts in the perfusion fluids. Extracellular ions and potassium also perfused readily from the liver of the control dog. Liver perfusions by our technique, therefore, do not give an accurate indication of extracellular space, since extravascular and even intracellular components are removed while blood cells are still being washed out.

DISCUSSION

The data presented above give no indication that there is any consistent shift of water or electrolytes into or out of cells in shock, barring what may occur locally in traumatized regions. This is made clear by the estimations of thiocyanate and radioactive sodium space in which any apparent slight increase in extracellular volume can probably be accounted for by local shifts in the traumatized regions; it is noteworthy that such shifts, when they occur, take place mainly within 2 hours after removal of clamps and ties at the time when local edema is manifest, rather than terminally when shock is greatest. Though the tissue analyses reported leave much to be hoped for as regards consistency, it appears clear that there is a marked loss of potassium and gain in sodium by the clamped and the tied muscles, while there is no change in the sodium and potassium of liver or other muscles within the rather wide limits of variation encountered. Any such shifts in extra-traumatic areas could only be demonstrated by a more refined technique. Even if they were found, they might be of doubtful application to human shock, since dogs apparently differ from rats in their handling of liver potassium in shock (13). It is of interest that certain workers have been unable to demonstrate increased capillary filtration, except in regions adjacent to trauma (22).

Areas of traumatization, particularly with the Duncan-Blalock press, show considerable edema; in fact, there is reason to believe that local loss of plasma is a most important factor in production of shock by such means. It is, therefore, worth while to consider whether the shift of tissue water to the extracellular compartment observed in these muscles may be wholly due to infiltration of the tissue with edema fluid. The calculations of intracellular potassium concentration in these tissues (Table III) and in K:N ratio show a decrease in cell potassium which is probably beyond the limits of experimental variation, and thus suggest that there is a loss of cell potassium, in addition to edema, amounting in the case of muscle injured by the Duncan-Blalock clamp to about 25 to 40 per cent. In muscle tying, the figures suggest that the loss is smaller. Our results confirm the conclusions of others (6) that the electrolyte changes in shock and in adrenal insufficiency differ fundamentally, although the plasma potassium is elevated in both conditions (6, 23).

SUMMARY

Extracellular space measurements and tissue and blood electrolyte determinations were made on dogs in which shock was produced by hemorrhage and by 3 procedures producing trauma or ischemia.

No evidence was found of significant changes in the extracellular space of the whole body with these procedures except that which occurred locally in traumatized areas.

Plasma sodium and chloride did not show any significant changes; increase in plasma potassium terminally, as reported by others, was confirmed.

Tissue analyses showed marked loss of potassium and gains of sodium by muscles which had been directly traumatized or tied to produce ischemia. This change was probably due in part to local intra- or extra-cellular edema and in part to net loss of potassium from injured cells.

Bibliography follows Paper VI of this series.

THE TOXIC FACTORS IN EXPERIMENTAL TRAUMATIC SHOCK.

III. SHOCK ACCOMPANYING MUSCLE ISCHEMIA AND LOSS OF VASCULAR FLUID¹

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A discussion of the hemodynamic factors involved in the production of traumatic shock has already been presented (24). It was concluded that on the basis of both clinical and experimental observations, local loss of vascular fluid into the traumatized areas must be considered the principal cause for the development of shock following injuries, but that other factors such as nociceptive nervous impulses and hypothetical toxins elaborated in the damaged tissue have not been ruled out as contributory elements in this process. This paper will be concerned with describing a further attempt to demonstrate the action of a toxic factor by the use of the triceps surae ligation procedure previously described (14).

The theory of traumatic toxemia has been based upon two types of experimental studies. One of these has consisted of the parenteral administration to normal animals of various extracts and products of both normal and damaged tissues with the development of a shock-like state in the recipient. The significance of experiments of this sort will be discussed subsequently (25).

A more direct approach to the problem consists in producing shock in animals by actual traumatizing procedures of such a nature as to permit measurement or control of other factors (fluid loss, neurogenic) and hence an evaluation of the possible etiologic rôle of toxins. Pioneer studies of this sort were carried out during the first World War (26, 27, 1). The findings were interpreted as indicating the operation of a humoral agent in the pathogenesis of shock and resulted at the time in a widespread acceptance of the theory of

traumatic toxemia. These conclusions, however, have since been considered as inconclusive because of incomplete estimation of the local fluid loss which probably occurred in their animals (28 to 31).

Recently, traumatic shock was induced in dogs by repeated hammering of an extremity, and local fluid loss was measured by a water displacement technique (32). They concluded that although local fluid loss was a major etiological factor, it alone in their experiments was insufficient to produce fatal shock. In their series of 20 dogs, however, the average local fluid loss was 3.4 per cent of the body weight and 80 per cent of the animals exhibited a local fluid loss of over 3 per cent of the body weight. These figures are of the same order of magnitude as the amounts of simple hemorrhage which most investigators find necessary to produce shock (33 to 41). Consideration should also be given to the fact that their measurements of effusion gave minimal values and that plasma loss might be more detrimental than loss of similar quantities of whole blood.

Some investigators have attempted to demonstrate the operation of toxic factors by cross circulation experiments of varying degrees of complexity (42 to 44). Although it is likely that toxic factors, if present, might exert some effect on the untraumatized animal of a cross-circulated pair, it is obvious that this animal would lose vascular fluid into the traumatized limb of the other. It is surprising that positive results with such experiments were not more frequent. Recognizing this difficulty, two groups have attempted to avoid it by substituting for cross circulation, repeated and equal cross transfusions of whole blood. The continuous exchange of equal quantities of blood between a traumatized and a normal dog with the occasional development of shock in

¹This is reprint No. 607 of the Cancer Commission of Harvard University.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts General Hospital.

the latter was interpreted as evidence for the action of a toxic factor arising in the traumatized region of the former (32). Since the local fluid loss is almost entirely plasma, while the cross transfusions are equivalent only in terms of whole blood, it is apparent that in these experiments as in the simple cross circulation experiments the normal animal continues to lose plasma into the traumatized limb of the other. Shock produced in normal dogs by cross transfusion with a heart-lung traumatized limb preparation was assumed to be due to the action of a toxin (45). In each of the four experiments reported there was a local loss of fluid of about one liter into the traumatized limb. How much of this came from the normal dog it is difficult to judge, since the authors fail to state the volume of blood in their reservoir. It could, however, have represented an appreciable plasma loss from this animal if the hematocrit of the blood infused into the latter was higher than that of the blood simultaneously withdrawn. Two things should be noted regarding these experiments; in many cases blood may have been traumatized, and in the cases cited the results of experiments have been variable.

Using a bandaging technique to minimize local fluid loss on sympathectomized and spinal animals, it has been concluded that there was evidence for the operation of some factor other than local fluid loss (46 to 48). Quite recently evidence has been presented that trauma plus ischemia, but not trauma alone, results in shock even when the reduction in blood volume incident to the local fluid loss is corrected continuously (4). Although no conclusion was drawn regarding toxic factors, it would be important to rule out possible neurogenic elements before these experiments are regarded as the good evidence which they appear to be for the operation of the products of damaged and ischemic tissues in the production of this type of shock. In summary, the evidence for the toxemic theory of shock, while suggestive, is based upon experiments where it has been difficult to control other causative mechanisms.

Nor is the evidence against the toxemic theory conclusive. Experiments in which local fluid loss is measured and found to be sufficient to cause shock (28, 29, 31, 49 to 51) were of great value in establishing the importance of this process in the pathogenesis of traumatic shock but do not

rule out the possible contributory rôle of other factors. It has been shown that when local fluid loss in regions of trauma is artificially restricted, shock does not occur (3, 4, 46). Although such experiments speak against the likelihood that factors other than fluid loss can cause shock, they do not rule out the possibility that such factors may facilitate or aggravate the effects of reduced blood volume. It is conceivable that there may be in traumatic shock certain humoral substances released from areas of tissue damage which have no specific effect on the intact circulatory system and could not in themselves cause shock or even a fall in blood pressure, yet by virtue of their effects on cell metabolism could play a rôle in lowering the resistance to blood loss or anoxia. Experiments in the past have not been designed either to establish or disprove the existence of such factors and for that reason the present work was undertaken. A localized area of tissue damage was produced in a series of dogs, and these animals together with a group of untraumatized controls were subjected to controlled hemorrhage. It was felt that by comparing the incidence of shock in the two groups, the possible rôle of factors other than vascular fluid loss could be evaluated.

METHODS

Mongrel street dogs were used in all experiments. They were anesthetized with sodium pentobarbital in an initial dose of 30 mgm. per kgm. of body weight and subsequent doses of 30 mgm. as required. The muscle ligations were performed according to the procedure described in the first paper of this series (14) but without sterile precautions. Rubber ligatures were kept in place for 5 hours and then released. During this interval the animals were bled from a catheter inserted through the external jugular vein into the right auricle, in steps of 1 or 0.5 per cent of body weight at about 30-minute intervals until the total bleeding reached a predetermined amount from 0 to 3 per cent of body weight. Control animals were not operated upon but were bled corresponding amounts at the same time.

Blood pressures were taken at frequent intervals by means of a needle inserted into the femoral artery and connected to a mercury manometer. Determinations of venous oxygen saturations and hemoglobin concentrations were made on mixed venous blood obtained from the right auricle. Blood loss incidental to the operation and to the blood pressure measurements was estimated by saving all sponges used and determining the hemoglobin content of an aqueous extract.

The release of the muscle ligature is immediately followed by a progressive swelling of the limb which begins

in the gastrocnemius and spreads throughout the leg from the thigh to the ankle. In some dogs an attempt was made to measure the amount of this local fluid loss in the following manner.

On the medial aspect of the closely-shaved leg an ink line is drawn longitudinally bisecting this surface from the groin to the internal malleolus (Figure 1). At 8 equidistant points along this line, and in each case perpendicular to it, circumferences are drawn about the limb. By treating the segment of limb between the 2 extreme

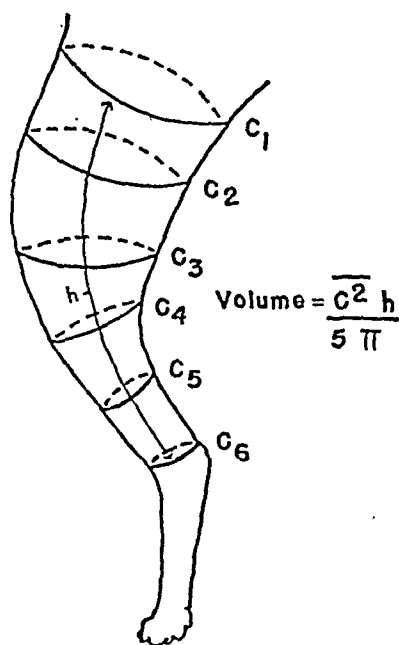


FIG. 1. ILLUSTRATION OF THE METHOD USED IN CALCULATING FLUID LOSS INTO A LIMB

circumferences as a flexed, truncated, elliptical cone with 1 semi-axis twice the length of the other (an assumption which can be shown to fit the actual shape remarkably closely), it is possible to derive the volume of this segment in terms of the length of the longitudinal axis (h) and the mean of the squares of the several circumferences (\bar{c}^2) thus:

$$V = \frac{\bar{c}^2 h}{5\pi}$$

Calculation of the volume of a limb segment obtained by this method agreed within 2 per cent of that observed by water displacement. Measurement of the increase in volume of the limb by this technique is, however, minimal since only a large segment of the leg is included, and it is known that such fluid dissects its way not only throughout the limb but even into the flanks. To test the recovery of added fluid by this technique, known quantities of plasma were injected into the limb of a dead dog and the increase in volume determined. There was a consistent recovery of only 80 per cent of the injected fluid, the remainder having spread outside the area of measurement. Values for volume increase obtained by this tech-

nique have, therefore, been corrected by a factor of 1.25 in an attempt to approximate the true magnitude of the effusion. This technique was used because it permits frequent determinations of local fluid loss throughout the course of the experiment.

The animals operated upon were followed for at least 5 hours after the release of the ties and the controls for a corresponding length of time.

RESULTS AND DISCUSSION

The experimental data and results are presented in Tables I and II. The last column indicates the development or absence of shock, the criteria for which included a progressive decline of blood pressure to below 70 mm. Hg, and (where this was measured) a consistently depressed mixed venous oxygen saturation.

There were 41 dogs with muscle ties plus hemorrhage and 23 dogs with hemorrhage alone. The operative blood loss was measured in 23 of the dogs operated upon and 11 controls which were only bled. The average operative blood loss in the 23 operated dogs amounted to 0.5 per cent of the body weight, and this figure was used in estimating the total blood loss of those dogs in which the operative loss was not directly determined. Similarly, the average incidental blood loss in the 11 dogs with hemorrhage alone amounted to 0.2 per cent of body weight and this was added to the purposeful blood loss of other such animals in which it was not measured.

The effusion into the operated limbs was measured in 7 dogs. In these dogs the measured effusion was multiplied by 1.25 to obtain an estimate of the total effusion as described above. The average measured effusion was 1.3 per cent \pm 0.2 per cent of the body weight, and this figure multiplied by 1.25, or 1.6 per cent, was used in estimating the total fluid loss in those dogs in which the effusion was not directly measured.

The calculation gives a value for the effusion which is almost certainly minimal. Any fluid dissecting its way superiorly into the flank or along the back was not measured by the technique employed. This has been by no means a negligible amount in similar experiments (30, 52). In addition, not inconsiderable amounts of unmeasured fluid dissected downward into the ariles which exhibited marked pitting edema in all of all instances. Moreover, no attempt has been made to estimate the equivalence of the traumatic

KETY, NATHANSON, NUTT, POPE, ZAMECNIK, AUB, AND BRUES

TABLE I

Observations on dogs with muscle-tie and graded hemorrhage

NUTT, POPE, ZAMECNIK, AUB, AND BRUES													
TABLE I													
Observations on dogs with muscle-tic and graded hemorrhage													
Number of dog	Weight kgm.	Blood loss			Vascular fluid loss		Blood pressure		Venous O ₂		Hemoglobin		Shock
		Operative	Sam- ples	Pur- posful	Effu- sion *	Total	Initial	Final	Initial	Final	Initial	Final	
20	16.0	2.1	1.2	0	1.6	4.9	130	44			10.0	13.8	+
21	20.5	0.5	1.0	0	1.6	3.1	148	42			9.1	13.4	+
22	13.5	0.5	1.6	0	1.6	3.7	156	50			9.1	13.3	+
26	20.0	0.5	1.0	0	1.6	3.1	165	54			17.5	19.5	+
30	11.0	0.5	2.2	0	1.6	4.3	108	60			9.0	14.6	+
35	24.0	0.5	1.5	0	1.6	3.6	190	40			17.2	22.5	+
38	8.5	0.5	1.2	0	1.6	2.8	130	54			10.0	9.3	+
39	18.0	0.5	0.7	0	1.6	3.3	130	40			15.7	16.0	+
41	19.0	0.5	2.2	0	1.6	2.8	150	40			14.7	20.1	+
48	13.0	0.5	0	0	1.6	5.8	106	70	5				+
49	11.0	0.5	0	0	1.6	2.1	100	66	40				+
55	13.5	2.0	0	0	1.6	2.1	35	69	22				+
56	15.5	0.5	0	0	1.6	2.1	60						+
57	13.0	0.5	0	0	1.6	2.1	100						+
58	10.5	0.5	0	0	1.6	3.9	110						+
59	20.0	0.5	0	0	1.6	2.1	140						+
60	19.5	0.5	0	0	1.6	3.4	120						+
61	6.5	0.5	0	0	1.6	3.8	130						+
62	16.5	0.5	0	0	1.6	2.7	120						+
63	19.5	0.6	0	1.8	1.6	3.1	154	64					+
65	21.0	1.1	0.2	0	1.6	3.4	80	82	12				+
66	15.5	0.2	0.1	1.0	1.6	3.5	114	57	38	13.3			+
68	18.0	0.2	0.1	1.0	1.6	4.0	117	54	72	12.3	21.4		+
71	17.5	0.3	0.2	0.8	1.6	4.0	122	64	18	16.6	16.2		+
72	17.5	0.2	0.2	0.7	1.6	4.0	90	36	63	15.8	17.6		+
74	13.0	0.2	0.1	1.0	1.6	3.6	100	55	33	18.4	15.6		+
75	19.0	0.2	0.2	1.5	1.6	4.7	91	78	39	16.5	21.0		+
77	23.0	0.2	0.2	2.0	1.6	4.1	54	84	9	17.9	15.5		+
78	20.0	0.4	0.2	1.3	1.6	4.1	36	64	40	9.4	20.4		+
80	21.0	0.3	0.2	2.0	1.6	4.1	50	74	58	12.9	13.9		+
81	19.0	0.2	0.1	1.5	1.6	4.1	110	30	32	14.7	16.9		+
83	22.0	0.1	0.1	2.8	1.6	4.4	60	74	12	14.8	20.2		+
84	22.0	0.1	0.1	2.3	1.6	4.1	69	42	75	11.8	18.7		+
86	18.0	0.1	0.1	2.3	1.6	4.1	53	69	80	13.2	12.7		+
87	22.0	0.1	0.1	1.0	1.8	2.5	110	70	34	7.5	16.7		+
89	21.0	0.4	0.2	0.4	2.0	2.1	67	34	14	16.3	12.1		+
90	14.0	0.5	0.3	0.4	2.0	2.0	18	55	14.2	17.0	15.6		+
92	25.0	0.6	0.2	0.1	2.0	2.0	23	60	10.7	14.8	18.4		+
93	12.5	0.7	0.2	0.1	2.0	2.0	62	43	11.7	12.8	21.5		+
95	18.0	0.2	0.3	0.1	2.0	2.0	50	56	10.6	15.2	15.0		+
96	16.5	0.4	0.2	0	2.0	2.0	82	61	9.8	20.5	20.5		+
									11.7	16.0	13.3		+

* Effusion was actually measured in numbers 87 to 96. Figures in other cases are estimated (see text).

Effusion in terms of whole blood loss. The total protein of this effusion fluid has been found to average 4.2 grams per 100 ml. which is 70% of the value of the corresponding whole blood protein concentrations. The loss from the blood is therefore 1.4 grams per 100 ml. of blood.

culatory embarras

* Effusion was actually measured in numbers 87 to 96. Figures in other cases are estimated (see text).

effusion in terms of whole blood loss. The total protein of this effusion fluid has been found to average 4.2 grams per 100 ml. which is 70 per cent of the value of the corresponding plasma protein concentrations. It seems highly probable that the loss from the circulation of a given volume of plasma is more detrimental than that of the same volume of whole blood since the former contains all of the osmotically-active constituents of the latter, while the proportionally greater hemoconcentration may result in even further cir-

culatory embarrassment because of the greater increase of blood viscosity (53). The incidence of shock with graded amounts of total fluid loss in both the operated and control animals (Table III) demonstrates no significant difference between the two groups. These dogs were bled fixed amounts, and the incidence of shock at each degree of total fluid loss determined; whereas most investigators studying the effects of hemorrhage have bled dogs until they went into shock, a difference in technique which ought

TABLE II
Observations on dogs with hemorrhage alone

Number of dog	Weight	Blood loss				Hemoglobin		Venous O ₂		Blood pressure		Shock
		Operative	Samples	Purposeful	Total	Initial	Final	Initial	Final	Initial	Final	
	kgm.		per cent of body weight			grams per cent		per cent of saturation		mm. Hg		
1	17.5	0.2		4.6	4.8					104	34	+
2	19.0	0.2		2.1	2.3	15.4	15.4			95	20	+
3	17.5	0.2		4.4	4.6	11.0	9.9			120	30	+
4	10.0	0.2		4.8	5.0	11.6	5.5			110	46	+
5	14.5	0.2		5.9	6.1	14.3	9.0			120	28	+
6	10.5	0.2		5.0	5.2	17.4	16.0			120	36	+
7	20.0	0.2		5.7	5.9	12.3	15.1			118	56	+
8	16.5	0.2		5.9	6.1	9.7	13.8			128	36	+
9	15.5	0.2	1.8	0	2.0	12.0	13.1			110	100	0
10	12.0	0.2		3.6	3.8	12.6	12.4			142	68	+
12	22.5	0.2		3.4	3.6	16.0	13.3			148	35	+
18	21.0	0.2	1.4	0	1.6	12.7	17.4			160	138	0
67	18.0	0.1	0.1	2.0	2.2	13.9	15.9	60	37	136	50	+
70	17.5	0.1	0.2	1.0	1.3	16.2	20.2	64	37	128	132	0
73	15.5	0.1	0.2	1.5	1.8	14.1	19.2	70	29	146	112	0
76	21.0	0.2	0.2	2.0	2.4	11.2	14.6	84	42	150	125	0
79	20.5	0.2	0.1	1.3	1.6	17.2	16.1	90	75	163	120	0
82	17.0	0.1	0	1.5	1.6					170	45	+
85	12.0	0.5	0.2	2.3	3.0	13.9	17.7	74	27	140	38	+
88	19.0	0.1	0.1	2.3	2.5	16.7	16.3	65	50	160	116	0
91	15.0	0.3	0.2	2.0	2.5	13.8	15.2	41	60	160	120	0
94	32.5	0.7	0.1	1.7	2.5	12.4	16.7	32	73	165	94	0
97	18.0	0.2	0.2	3.0	3.4	13.2	12.1	92	54	155	157	0

to result in a somewhat higher average blood loss in the latter type of experiments. In spite of this difference, our results in both the operated and control series are quite comparable to most values obtained by other investigators for the amounts of blood loss necessary for the production of shock in anesthetized dogs (33 to 41). The relatively high incidence of shock (35 per cent) in dogs with a total fluid loss of from 2 to 3 per cent of the body weight is not remarkable in view of the repeated observations of many authors that some dogs are unusually susceptible to hemorrhage. Thus shock has been produced by bleeding dogs from 1 to 2.2 per cent of body weight (34, 5, 37, 39).

TABLE III
Incidence of shock with graded vascular fluid loss

Total vascular fluid loss	Incidence of shock			
	With muscle-tie		Control	
per cent of body weight		per cent		per cent
Less than 3.0	5/14	36	4/13	31
3.1 to 4.0	12/17	71	2/3	67
4.1 to 5.0	7/9	78	3/3	100
Greater than 5.0	1/1	100	4/4	100

There is, therefore, no evidence that under these experimental conditions any agent derived from ischemic tissues is acting generally either to enhance the severity of the shock state directly, or to sensitize the animal to the noxious effects of vascular fluid loss.

Before drawing any general conclusion, however, it is necessary to note the limitations of these experiments. (1) No effort was made to control possible neurogenic stimuli from the traumatized areas except that which is incident to the use of barbiturate anesthesia. However, any consequences of nervous stimuli should have resulted in a difference between the two series of animals. (2) It is possible that too small a mass of tissue was rendered ischemic, and that had such damage been greater in extent, noxious influences might have revealed themselves. (3) In these experiments, ischemia with only a relatively minor degree of actual trauma was produced. It might well be that similar experiments in combination with severe traumatization would reveal the presence of toxic substances derived from the traumatized and ischemic area. Indeed a recent study (24) furnishes evidence suggesting that this may be so. (4) Finally, it is possible that the bleeding

in these studies may have been too rapid, and that the experiments were not prolonged sufficiently to bring out more subtle differences in susceptibility to vascular fluid loss. Nevertheless, these experiments fail to demonstrate the presence in the general circulation of any noxious substance liberated from ischemic muscle, which accentuates the appearance of shock. Local effects which might influence the amount of traumatic effusion cannot be analyzed in these observations.

SUMMARY

A comparison of the incidence of shock caused by graded amounts of vascular fluid loss has been

made in animals subjected to muscle ischemia and in a series of normal controls. By this technique, no evidence has been obtained for the production in an ischemic region of any agent capable of lowering the general resistance of the animal to the loss of vascular fluid. Following a period of ischemia to the triceps surae muscle groups, there was a loss of fluid into the legs, averaging 1.6 per cent of the body weight. The evidence indicates, therefore, that the important shock-producing factor in muscle anoxia results from influences which increase the local loss of vascular fluid without producing a generalized toxic action.

Bibliography follows Paper VI of this series.

THE TOXIC FACTORS IN EXPERIMENTAL TRAUMATIC SHOCK.

IV. THE EFFECTS OF THE INTRAVENOUS INJECTION OF THE EFFUSION FROM ISCHEMIC MUSCLE¹

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The fundamental hemodynamic disturbances which characterize traumatic shock may be initiated by at least 3 different factors: gross hemorrhage or loss of plasma into a traumatized region, neurogenic and psychogenic elements, and the absorption of toxic materials from traumatized regions. Of these etiologic factors, the local loss of vascular fluid is generally conceded to be of greatest importance (3, 28, 31, 54), while the others can be at best but contributory.

Since the original classical experiments of Cannon and Bayliss (1), the theory of traumatic toxemia has been furthered by two types of experimental evidence. In one, a state more or less resembling clinical shock is produced in animals by the parenteral administration of various extracts of normal or traumatized tissues or by the intraperitoneal implantation of tissue. In the other, attempts are made in various ways to control or limit local fluid loss and neurogenic factors in experimental traumatic shock and to demonstrate that an additional factor, presumably traumatic toxemia, is necessary to explain the results. This approach has been considered in another publication (55) and the present discussion will concern itself only with the first type of experiment.

Many experiments have been reported in which extracts of various tissues were found to have definite vasodepressive or shock-producing properties (56 to 61). Certain techniques (62, 63) consist of introducing into the peritoneal cavity of dogs whole or pulped liver or muscle. Observed regularly were hemoconcentration, circulatory col-

lapse, and death within 24 hours. Furthermore, at autopsy the viscera consistently showed congestion, edema, and gastrointestinal hemorrhage. More recently, shock was observed following the parenteral injection of extract of striated muscle, and adenosine triphosphate was implicated as the constituent responsible for these effects (64).

In the experiments cited, there have been no bacteriologic studies, and in only a few has any attempt been made to prevent bacterial contamination during the course of the experiment. The inability to reproduce the results of some of these experiments, when strict aseptic technique was used (65), raises the question as to how many of the positive results reported may have been due to extraneous bacterial contamination.

In many of the cited experiments the criteria of shock do not appear conclusive. They consist either of death of the animal, certain autopsy findings, a fall in blood pressure, or combinations of the three. In some experiments, hematocrit studies have been done in addition and these have usually shown a marked hemoconcentration. Death and hypotension are hardly pathognomonic of shock, while recent studies in clinical traumatic shock without evident hemorrhage have revealed a falling hematocrit (66).

Dosage, rate, and route of administration are of paramount importance in evaluating the toxicity of any substance, yet these factors appear to have received insufficient attention in experiments on the toxicity of tissue extracts. If one may convert to clinical terms the experiments of one investigator (64), it would require that a large part of the muscle tissue of the body be ground, extracted, and administered in one dose in order for shock to follow. It would be desirable to determine the rate at which substances might be absorbed from a traumatized region and to reproduce that rate

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in the administration of tissue extracts. It is not sufficient to demonstrate that substances obtainable from traumatized tissue are toxic; it must be clearly shown that they are toxic in the form, in the dose, and at the rate of absorption which might conceivably occur in clinical traumatic shock.

In view of these considerations, the remarkable thing about the injection of tissue products is not that they are toxic but that they are so often found to be innocuous. In recent experiments with lymph from traumatized limbs, noxious effects were produced in recipient animals in only 30 per cent of the trials (67). In one series of recipients, normal lymph was found to possess toxic properties in 50 per cent of cases (68). The consistently negative results which have been obtained by another group when bacterial contamination is prevented have already been mentioned (65). Extracts of traumatized limbs were found to cause no blood pressure fall or any untoward effects when administered intravenously in excessive dosage (69). This observation is particularly difficult to reconcile with the thesis that significant quantities of shock-producing factors may be liberated from traumatized regions.

The theory of traumatic toxemia can, therefore, hardly be called established, and it seems that further experimental work is warranted. The muscle ligation technique previously described (14) affords a method for obtaining in large quantities the fluid which effuses from an area damaged by prolonged ischemia. Chemical examination of this fluid (70) indicates that it represents plasma which passes through the damaged capillaries and muscle tissue carrying with it products of cellular disintegration. It seemed reasonable that toxic factors if they exist should be present in such fluid and that a pharmacodynamic study of the effects of this fluid on intravenous administration might be of value.

METHODS

The operation of muscle ligation, release after 5 hours, and fluid collection as described previously (14) were performed with sterile precautions. The fluid which collected during the 5 hours following release of the ties was centrifugalized and the supernatant fraction frozen and preserved on carbon dioxide snow until used. Before administration, the fluid was thawed, filtered through a few layers of gauze, warmed to room temperature, and

administered intravenously over a period of 15 to 30 minutes. The dose given which approximated 14.7 ml. per kgm. exceeded slightly the average output (12 ml. per kgm.) of the 35 donor animals in this series. The animals used were mongrel dogs in apparently normal health, anesthetized with sodium pentobarbital. Blood pressure was recorded continuously by a mercury manometer connected to a carotid cannula containing dilute heparin or 2.5 per cent sodium citrate solution. Cardiac output was determined at intervals using the Fick principle as first used in shock by two investigators (71). Mixed venous blood was obtained from a catheter passed down one external jugular vein with the tip either in or at the level of the right auricle. Arterial blood was taken from a femoral artery by puncture. The bloods were collected and preserved under oil, and oxygen content was determined by the manometric method of Van Slyke and Neill. Oxygen consumption was measured by a calibrated recording spirometer connected to a tracheal cannula. Carbon dioxide was removed by soda lime in the system. Relative total peripheral resistance was calculated from the relationship:

$$\text{Relative total peripheral resistance} = \frac{\text{Mean arterial B. P.}}{\text{Cardiac output}}$$

No attempt was made to calculate the resistance in absolute units since only changes from control values were desired, but in order to permit better comparison between animals of different weights, cardiac outputs were substituted in the formula in terms of liters per minute per kgm. of body weight.

Mixed venous blood hemoglobin concentration was determined photocolometrically. When used to measure the trend of hemoconcentration in a given experiment this determination possesses the advantage over erythrocyte hematocrits in that it is independent of changes in erythrocyte size and of such variables as speed and duration of centrifugation, plasma viscosity, and specific gravity. Its use as a gross index of plasma loss seems warranted, although, in common with the great vessel hematocrit, it cannot be said to reflect quantitatively the average body hematocrit.

The recipient animals were observed for a period of at least 5 hours after the injection of fluid. If throughout that time no significant change had occurred in the condition of the animal, the experiment was terminated. Where any change had occurred, observations were continued until the death of the animal. Because of the possibility that the plasma proteins present in the injected fluid might augment the blood volume of a normal recipient and either mask any possible toxic effects or cause harm in itself by mere plethora, in 10 experiments the fluid was reinjected at the end of the collection period into the animal which had produced it. It thus merely partially replaced plasma volume already lost. The results were in no way different from those in which the fluid was administered to other recipients. Gross and microscopic examination of the viscera were made in the majority of animals. Bacteriologic studies which were made on the fluids are reported in another communication (11).

RESULTS AND DISCUSSION

The results are presented in Tables I and II. They divide themselves sharply into 2 groups. In 1 (Table I), the injection of the fluid resulted in a progressive decline in blood pressure and cardiac output, death of the animal usually within 5 hours, and consistent autopsy findings. These are the criteria of shock used in our experiments. The characteristic fall of blood pressure was interesting for it was invariably a distinctly delayed reaction. Some fluids had a mild vasodepressor action which caused a prompt and short fall of blood pressure which rapidly disappeared. The blood pressure then usually rose nearly to the control level. After a delay of 30 to 45 minutes, the shock-producing effect became manifest in a gradual progressive fall in blood pressure. Figure 1 illustrates the blood pressure response of an animal following injection of muscle exudate. In a few instances the rate of blood flow through one femoral artery was measured by means of a modification of an air bubble flow meter (72). In animals showing the other criteria of shock following the intravenous injection of muscle exudate, a significant reduction in the peripheral blood

flow was observed, consistent with the reduction in cardiac output.

In the other group (Table II), none of these effects were observed. Even though the results described in the previous paper of this series indicate the dominance of fluid loss, this is good evidence for an additional inconstant toxic factor in muscle exudate which has the effect of producing shock, in spite of increasing the plasma volume by roughly 25 per cent. The effect was obtained consistently in the pooled fluids (number 9) accumulated from 9 dogs, indicating that the effect does not depend on individual sensitivity of certain animals to the toxin.

Examination of the possible sequence of events in the first group of animals indicates that the rapid and progressive decrease in cardiac output precedes, and is the probable cause of, the drop in blood pressure. The progressive increase in total peripheral resistance may be the result of a compensatory peripheral vasoconstriction. The reduction in cardiac output, however, is not specifically explained by the data available. It could result from either an intrinsic myocardial change or a decrease in venous return to the heart. The

TABLE I
Positive muscle exudate assays

Donor dog number	Fluid number	Recipient dog number	Weight	Dose	Blood pressure			Cardiac output			Relative total peripheral resistance			Blood hemoglobin		
					I	II	III	I	II	III	I	II	III	I	II	III
			kgm.	ml. per kgm.	mm. Hg			liters per kgm. per minute						grams per 100 ml.		
Pool*	9**	122	11.5	15.0	134	110	60	0.13	0.19	0.03	1040	580	2000			
Pool	9	123	5.0	15.0	120	75	60	0.10	0.02	0.02	1200	3750	3000	15.3	15.7	19.2
Pool	9 (dial.)	128	7.5	25.0	130	100	34	0.13	0.05	0.03	1000	2000	1133	12.3	10.0	15.6
Pool	9 (dial.)	129	6.5	27.0	138	124	45	0.12	0.06	0.02	1151	2067	2250	13.8	14.4	13.8
Pool	9 (prot. frac.)	132	8.0	45.0	140	72	40	0.15	0.06	0.01	935	1200	4000	16.4	17.2	18.9
Pool	9	136	9.0	24.0	160	138	104	0.13	0.15	0.04	1230	917	2600	19.8	15.2	17.2
148*	17	149	9.8	5.0	145	50	65							18.5	18.5	17.8
151	19	151	29.0	9.7	130	130	32	0.05	0.04	0.02	4333	3250	1600	16.7	17.2	18.3
172	24	173	12.0	15.0	142	135	65	0.16	0.08	0.05	888	1688	3250	13.1	11.5	11.7
180*	28	182	6.0	15.0	110	78	30	0.34	0.08	0.02	324	975	1500			
181	29	183	5.8	15.0	108	55	35	0.12	0.05	0.05	900	1100	700	12.0	8.5	7.2
148*	148	148	17.5	11.4	130	100	60									
154*	154	154	23.5	22.0	120	120	50									
Average			11.5	18.9***	130	90	54				1500	1753	2203	15.3	14.3	15.5

* Data incomplete.

** Pooled fluid 9 is discussed in detail in the following paper of this series (23). Following dialysis, dial., the non-dialyzable portion retained its activity, and the protein fraction (prot. frac.) which is eluted out from 0.2 to 0.7% solution with ammonium sulfate was the most highly active. The dosage of these fluids has been calculated from the original fluid involved, and does not take into account losses in toxicity which must have occurred during dialysis and fractionation.

*** Average dose emitting fractionation experiments, 14.7 ml. per kgm.

I Before injection.

II Immediately after injection.

III Five to six hours.

TABLE II
Negative muscle exudate assays

Donor dog	Fluid number	Recip- ient dog number	Weight	Dose	Blood pressure			Cardiac output			Relative total peripheral resistance			Blood hemoglobin		
					I	II	III	I	II	III	I	II	III	I	II	III
			kgm.	ml. per kgm.	mm. Hg			liters per kgm. per minute						grams per 100 ml.		
135	10	135	20.0	14.8	94	118	92	0.07	0.07	0.15	1345	1690	614	13.8	13.1	14.8
137	11	137	17.5	11.4	108	124	130	0.08	0.16	0.16	1350	775	813	14.8	14.8	14.6
138	12	138	20.0	15.0	127	123	132	0.10	0.04	0.04	1270	3070	3300	16.7	14.2	13.6
142	13	142	20.5	7.0	100	137	87	0.05	0.08	0.04	2000	1713	2178	14.6	15.9	17.5
143	14	143	16.0	5.0	102	90	82	0.10	0.12	0.09	1020	750	1000	12.4	12.5	12.6
145	15	145	19.5	10.5	120	130	98	0.08	0.14	0.14	1500	930	700	17.7	18.3	18.2
147	16	147	20.0	6.7	100	142	108	0.12	0.22	0.11	834	646	983	15.2	15.2	15.4
151	19	152	10.0	15.0	95	104	92	0.03	0.09	0.03	3180	1159	3060	15.4	12.4	15.1
153	20	153	27.5	14.6	120	120	135	0.03	0.04	0.04	4000	3000	3380	15.2	14.6	13.8
166	22	167	6.0	15.0	160	95	140	0.28	0.07	0.30	672	1360	467	13.8	13.8	14.0
168	23	169	8.5	15.0	130	130	130	0.21	0.19	0.21	620	685	620	13.1	12.7	13.0
171	25	174	13.0	3.0	136	60	125	0.05	0.05	0.13	2730	1200	963	15.5	11.6	12.3
171*	25	175	6.8	13.0	160	132	142							12.8	11.2	11.9
176	26	178	7.8	9.0	132	125	118	0.18	0.15	0.14	734	834	842	11.7	11.7	11.2
177	27	179	6.0	15.0	95	93	130	0.13	0.23	0.17	730	405	765	9.6	7.2	8.0
214-215	31	216	6.8	13.5	144	90	125	0.40	0.09	0.13	360	1000	963	15.2	14.4	16.7
217-218	32	219	7.3	15.0	160	154	155	0.44	0.55	0.18	364	280	863	12.0	13.6	12.7
221-222	33	223	5.3	15.0	135	120	135	0.34	0.17	0.15	399	710	900	14.2	12.7	13.4
226-227*	34	228	5.8	14.0	130	130	120	0.19	0.14	0.10	684	927	1200			
Average			12.9	12.0	128	116	120	0.16	0.14	0.13	1320	1180	1315	14.1	13.3	13.8

* Data incomplete. I Before injection. II Immediately after injection. III Final or 5 hours.

latter change could in turn be due either to a loss in blood volume through capillary leakage or a pooling of the blood in dilated vascular beds. The absence of any significant hemoconcentration, however, is evidence against generalized capillary leakage of plasma as an initiating factor in these experiments. In this respect alone do these animals differ from those described by Moon (63); there is, however, a likely explanation for the hemoconcentration which he observed in a large part of his experiments. His experiments consisted of introducing a weighed amount of muscle pulp plus 100 ml. of saline into the peritoneal

cavity of dogs and subsequently measuring the fluid recovered from the peritoneum at autopsy. While the volume of exudate did not increase much in his observations, it is highly likely that the injected saline solution was replaced by a solution high in plasma proteins. The loss of plasma proteins into the peritoneum may well account for the hemoconcentration. In our observations, the reverse situation holds for we were diluting plasma with an intravenous injection of fluid high in proteins.

The post-mortem appearance of the animals in Group I was similar to that in Moon's experi-

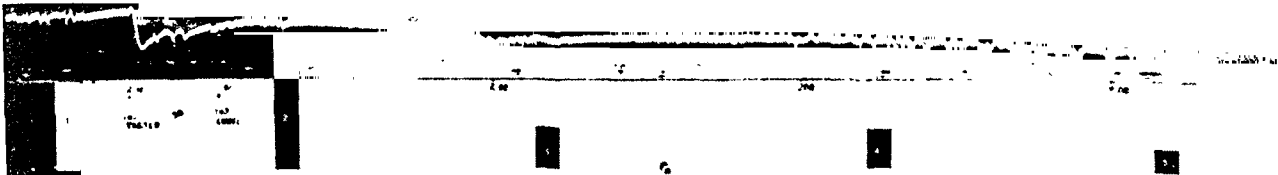


FIG. 1. KYMOGRAPH RECORD ILLUSTRATING THE BLOOD PRESSURE OF AN ANIMAL FOLLOWING INJECTION OF MUSCLE EXUDATES

The white blocks indicate cardiac outputs taken at various times during the course of the experiment. The first cardiac output taken after administration of the toxin (Number 2) is (in this case) increased, although such was not usually the case. The blood pressure response is a typical one. Cardiac output values are as follows, expressed in liters per minute: Number 1—1.47, 2—2.22, 3—0.69, 4—0.60, 5—0.35.

ments. There was intense visceral congestion, especially of the liver, together with moderate to marked hemorrhage into the intestinal lumen. This began abruptly at the pylorus, was most marked in the duodenum, and diminished distally.

A consideration of the proportion of positive to negative results in these experiments is illuminating. Of the total of 32 fluids studied, only 9 (or 28 per cent) were found to possess significant toxic properties, while the remaining 72 per cent were apparently innocuous.²

If the 9 toxic fluids derived their properties from noxious products of tissue breakdown, it is difficult to see how the other 23 specimens could have failed to contain these substances. All the muscles were subjected to the same degree and duration of ischemia and all the fluids were high in protein content indicating the degree of local capillary damage. Moreover both toxic and non-toxic fluids revealed similar electrolyte composition and the same concentration of proteolytic enzymes (70) indicating similar degrees of cellular breakdown. These considerations and the very inconstancy of positive results from fairly rapid intravenous injection suggest that the positive effects are due to inconstant factors which are not present in all muscles, but are probably an extraneous complication introduced into some experiments.

The possibility presents itself that an explanation for the positive effects in one-fourth of these fluids lies in bacterial contamination. The results of a detailed study of the number and types of

organisms present in these fluids appears elsewhere (11), and the evidence there presented clearly demonstrates a correlation between the toxicity of a fluid and its bacterial content.

Since the majority of these fluids yielded no evidence of a shock producing factor, it appears warranted to conclude that the usual metabolic breakdown products in anoxic muscles do not produce the vascular changes which precede shock. In our muscle ligation technique, the circulation in the anoxic muscles is usually well reestablished, so that diffusible toxic elements should be present in the circulation as a whole, as well as in the muscle exudate. However, this factor is controlled by the similar results which were obtained when fluids were reinjected into the original donor, as when given to recipient dogs.

SUMMARY

1. The exudate which accumulates after muscle anoxia was collected and injected into the same or recipient dogs.

2. In three-quarters of such observations, no evidence of shock was observed, while in one-quarter of the animals shock was produced by this technique and caused death.

3. The inconstancy of the presence of this toxic factor suggests it is an extraneous agent, not present in the usual cellular constituents and metabolic products found in all muscle exudates. Since the exudates are invariably contaminated with bacteria, it is thought that the toxin may be bacterial in origin.

² Table II does not include the earlier experiments in which the cardiac output was not measured.

THE TOXIC FACTORS IN EXPERIMENTAL TRAUMATIC SHOCK. V. CHEMICAL AND ENZYMATIC PROPERTIES OF MUSCLE EXUDATE¹

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Studies of the pharmacologic effects of exudates from traumatized limbs have been made by several workers (30, 69). Detailed studies have been lacking, however, on the chemical nature of exudates from anoxic or traumatized tissues. The technique of muscle tying previously described (14) has afforded an opportunity for collecting the exudate from a well-circumscribed set of muscles after a period of anoxia and trauma. This exudate, in amounts which ranged up to 480 ml., accumulated in the first 5½ hours after release of a constricting band which had produced anoxia for 5 hours.

We have studied, therefore, in some detail the chemical properties of muscle exudates obtained from single animals, as well as the properties of a pooled collection of muscle exudates from a group of 9 dogs. The central problem which has concerned us has been to determine the type of substance responsible for the toxic effects of certain muscle exudates.

MATERIALS AND METHODS²

All animals were anesthetized as described previously (14). In the enzymatic experiments a modification of the Grassmann-Heyde (73) titration technique was used, 0.2 ml. aliquots of test solution being titrated against 0.01 N alcoholic potassium hydroxide in a 90 per cent alcoholic medium, with 1:10,000 thymolphthalein as an indicator. It was necessary in some cases to titrate aliquots of test solution containing up to 3 mgm. protein nitrogen per ml. In these instances, a heavy protein precipitate appeared in the 90 per cent alcoholic titration medium, and the resultant turbidity interfered with the precision of the end-

point determination. It was found possible to obtain a satisfactory endpoint color change, nevertheless, if 0.2 ml. of 1 per cent thymolphthalein in absolute ethanol was used as an indicator in a titration medium containing, in addition, 0.2 ml. of test solution and 1.8 ml. of absolute ethanol.

The ammonium sulfate fractionations were made in the following way. The ammonium sulfate solution of the desired concentration was placed in a 12-liter cylinder and was dialyzed overnight at 2.5° C. into a rotating cellophane bag containing the muscle exudate, a technique in principle similar to that used by McMeekin (74). The precipitate was resuspended in 1 per cent sodium chloride and was dialyzed in a rotating cellophane bag against frequent changes of 1 per cent sodium chloride over the following 24-hour period until the ammonium sulfate was thoroughly dialyzed out.

The preparation of the aminoexopeptidase from normal dog muscle was made as follows. Five hundred and seventy-five grams of thigh muscle were obtained from a freshly killed dog and were frozen immediately on carbon dioxide snow. One week later the muscle was thawed, ground, and extracted at 2.5° C. for 3 hours with 2 volumes of 1 per cent sodium chloride and in the presence of toluene. All further procedures were carried out in the cold room at 2.5° C. The preparation was squeezed through cheese cloth, then made 0.4 saturated with ammonium sulfate by careful addition of the solid salt. The pH was 6.0. After standing overnight, it was filtered through a Buchner funnel with Whatman number 1 paper and Hyflo Supercel. The filtrate was then made up to 0.7 saturation with ammonium sulfate and allowed to stand overnight. The preparation was filtered through a Buchner funnel with number 5 Whatman paper, and the precipitate was dissolved in 140 ml. of 1 per cent sodium chloride. After 2 days' dialysis against 1 per cent sodium chloride, the solution was tested against L-leucylglycylglycine and found to be active ($C=0.03$). This order of activity represents a 10-fold increase over that of crude muscle. Adjustment of the pH of the muscle extract to pH 4 resulted in a marked loss of enzymatic activity. Lesser concentrations of enzymatic activity were found in the precipitates between 0 and 0.4 saturation ($C=0.003$) and above 0.7 saturation ($C=0.0001$) with ammonium sulfate. The enzymatic activity, therefore, is precipitated out principally between 0.4 and 0.7 ammonium sulfate saturation.

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² See acknowledgments.

EXPERIMENTAL

As a first step in the problem the chemical anatomy of this muscle exudate was studied. The observations consist of (A) a study of the electrolyte pattern of the fluid, and (B) a study of the protein constituents of the fluid by means of (1) electrophoresis, (2) dialysis, (3) fractionation of the muscle exudate proteins, and (4) study of proteolytic enzymes.

The exudate as it drained from the muscles was light red in color, odorless, with a hematocrit around 1 per cent, a specific gravity of 1.018, and a tendency to form a very thin clot. The fluid was collected in sterile flasks surrounded by ice. Following centrifugation the clear, practically cell-free, supernatant fluid was used for further experiments. The supernatant fluid was frequently preserved on carbon dioxide snow between the time of collection and the time of administration to another animal without noticeable change in its pharmacologic action.

In spite of employment of sterile techniques in operation and collection, the fluid contained bacteria in all experiments in which a search was made for their presence. A study of the rôle of bacteria in the production of shock by these muscle exudates has already been reported from this laboratory (10, 75).

A. Electrolyte studies

Figure 1 shows the electrolyte composition of pooled muscle exudate (fluid number 9) as compared with the electrolyte content of normal dog serum and lymph as compiled by Drinker and Yoffey (76). The conventions used are those originated by Gamble (77). This pooled muscle exudate produced shock in 2 dogs when injected intravenously (15 ml. per kgm.).

Muscle exudate differs from serum in the following particulars:

1. The total electrolyte concentration in muscle exudate is lower.
2. The pH of muscle exudate is lower, ranging from 7.0 to 7.2 in individual exudates.
3. The total protein concentration of muscle exudate is lower.
4. There is considerable hemolyzed blood, from 2.5 per cent present in the muscle exudates studied, as evidenced by a cherry-red color and a hemoglobin concentration ranging from 0.3 to 0.8 grams per cent in different preparations. It is highly probable that myoglobin contributes to this color. No attempt has been made to distinguish between these 2 globins.
5. The potassium and inorganic phosphate ($\text{HPO}_4^{=}$) concentrations of muscle exudate are increased.

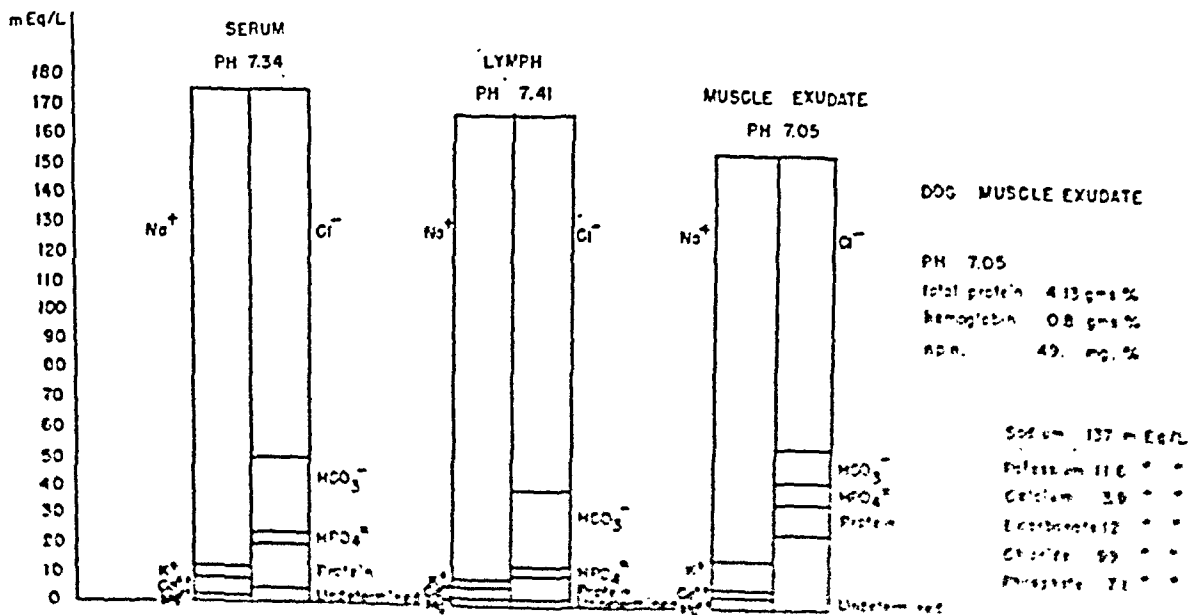


FIG. 1. COMPARISON OF THE ELECTROLYTE COMPOSITION OF DOG SERUM, LYMPH, AND MUSCLE EXUDATE

6. Muscle exudate contains less calcium than would be expected were it derived from plasma alone, even when the diminished protein content of muscle exudate is taken into account. Data on lymph (76) show calcium levels of 9 to 12 mgm. per cent, which are likewise higher than the muscle exudate level. In 2 individual experiments, the concentrations of calcium in the terminal serum were 11.5 and 10.9 mgm. per cent, while in the muscle exudates from the same animals they were 7.2 and 6.0 mgm. per cent, respectively.

7. The bicarbonate concentration of the muscle exudate is markedly decreased and the chloride concentration is somewhat diminished, resulting in an increase in total undetermined anions. In individual exudates the bicarbonate concentration has been as low as 6 millimoles per liter.

The findings of high potassium and inorganic phosphate concentrations indicate a breakdown of muscle cells, a leakage out of these intracellular ions, and perhaps decomposition of organic phosphate. They are consistent with analysis of potassium and chloride concentrations of fluid obtained from the subcutaneous tissue of a traumatized area (6). The decrease in the bicarbonate concentration and in total accountable anions in the presence of a lowered pH value points to an increase in undetermined organic acids, formed in part as a result of the anaerobic metabolism of the muscle.

B. Study of protein constituents

1. *Electrophoresis.* The electrophoretic patterns of a single muscle exudate, fluid number 117, have been compared with the patterns of plasma from the same dog before and after the muscle-tying and draining procedure.³ Electrophoresis was carried out in a phosphate buffer of pH 7.7, and ionic strength 0.2, at 1.5° C., over a 4-hour period, at a potential gradient of 4 volts per cm. Figure 2 shows tracings obtained from the electrophoretic patterns. It demonstrates that in the muscle exudate the fraction migrating with the same velocity as the gamma globulin fraction of plasma is increased considerably, and the alpha globulin fraction is decreased. The increase in the slowest-moving component suggests that in muscle

Dog 117

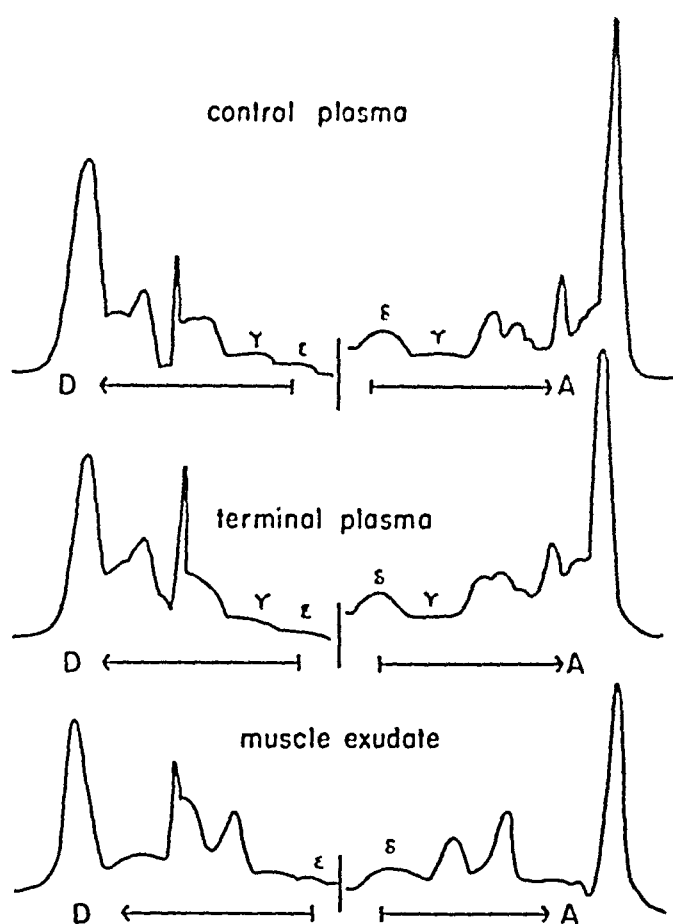


FIG. 2. TRACINGS OF ELECTROPHORETIC PATTERNS OF PLASMA AND MUSCLE EXUDATE

protein breakdown, proteins of electrophoretic properties similar to those of the gamma globulins of plasma make their appearance in the muscle exudate.

During the course of this experiment, there was a drop of plasma protein from a level of 7.0 grams per cent at the beginning to 5.0 grams per cent at the end of the experiment. The protein in the muscle exudate can conceivably be accounted for as coming largely from plasma.⁴ The electrophoretic patterns of plasma indicate a decrease in the relative concentration of albumin from the control plasma to the terminal plasma. The albumin:globulin ratio as calculated from the electrophoretic patterns changed from 0.9 to 0.7 during the course of the experiment.

⁴ Thus, in one experiment, even if the assumption is made that the plasma volume was not reduced during the course of the experiment, 25 grams of protein were lost from the plasma protein, and 14 grams of protein were found in the muscle exudate.

³ For these electrophoretic patterns and their interpretations the authors are indebted to Dr. Gertrude E. Perlmann, of the Massachusetts General Hospital.

2. *Dialysis studies.* After dialysis of the muscle exudate (fluid number 9) through Visking cellulose tubing, 0.0008 inch wall thickness, for 24 to 48 hours against changes of distilled water or 1 per cent sodium chloride at 2.5° C., it was found that the pharmacologically-active fraction was non-dialyzable. This non-dialyzable material was lyophilized in a Flosdorf-Mudd (78) apparatus, and the dry powder, re-dissolved in 1 per cent sodium chloride, was shown still to possess its shock-producing properties. This evidence rules out as possible major toxic agents a good many low molecular weight, dialyzable organic substances which might be present as a result of proteolysis or of the anaerobic metabolism of muscle. Although this evidence does not deny the possibility of adsorption of small molecular weight substances onto protein molecules, it does serve to focus attention on particles of large molecular size or aggregation.

3. *Fractionation studies.* A collection of muscle exudates (termed fluid number 9) was made from 9 dogs, using aseptic techniques, and freezing each exudate on carbon dioxide snow immediately after collection. The individual exudates were then thawed and pooled, amounting to 2100 ml. of fluid which was refrozen in 100 ml. lustroid containers. Assay of aliquots of this fluid in 2 dogs (numbers 122 and 123) resulted in typical shock (25). The remainder of the pooled fluid was subjected to fractionation procedures. Dialysis of a 150 ml. aliquot against 1 per cent sodium chloride for 18 hours at 2.5° C. revealed (dog 128) that the activity remained in the non-dialyzable portion. Another 150 ml. aliquot was dialyzed against 3 changes of 150 ml. each of distilled water for 18 hours at 2.5° C. Seven-eighths of the dialyzable material, therefore, was theoretically dialyzed out. Both the dialyzable and the non-dialyzable fractions were lyophilized, then 150 ml. of 1 per cent sodium chloride were added to each fraction which was then tested on a dog. The non-dialyzable material again produced shock (dog 129), while the dialyzable material did not (dog 126). An ammonium sulfate fractionation was done, therefore, on a 470 ml. aliquot to characterize more closely the shock-producing material. Table I summarizes this study. In order to determine whether the physiological activity of the pooled fluid decayed during storage on carbon

dioxide snow, an aliquot of the original pooled fluid was again tested (dog 136) and found to be active. In the protein fraction which salted out between 0.25 and 0.7 saturation there was (a) the characteristic physiological response of the animal, which died in shock approximately 5 hours after injection of this fraction, (b) 84 per cent of the protein nitrogen, and (c) all the detectable proteolytic activity. One may conclude that the toxic factors of the muscle exudate salt out in the α and β globulin and albumin range, and are probably not due to moities such as proteoses or peptones, which could be present in the muscle exudate as a result of muscle breakdown.

TABLE I
Fractionation of pooled muscle exudate
(number 9)

(NH ₄) ₂ SO ₄ concentration	Dog number	Result	Percentage of protein nitrogen recovered	Percentage of enzymatic activity recovered
0.25 saturation	131	No effect	4	0
0.25 to 0.70 saturation	132	Died in shock	84	100
0.70 to complete saturation	133	No effect	12	0

$$\frac{\text{enzymatic activity recovered}}{\text{enzymatic activity expected}} = 79 \text{ per cent.}$$

4. *Studies on proteolytic enzymes.* The evidence from the fractionation studies served to focus attention on that group of proteins which is highly active physiologically, the enzymes. Since it was impossible to study the 50-odd enzymes known to be present in muscle (79), 1 group of enzymes was selected for study in the hope that this group of enzymes might serve as a tracer for muscle enzymes as a whole in the fractionation and toxicity correlation studies.

The proteolytic enzymes in muscle exudate were selected for investigation on the hypothesis that such enzymes might be activated under conditions of anoxia and might either produce toxic substances *in situ* or prove toxic when liberated into the general circulation. Proteolytic enzymes have been described in muscle beginning as far back as 1905 (80 to 83).

In addition to testing for activity at pH 5, tests were made at other pH's, turning in some experiments up to pH 9. Table II shows the effect

TABLE II
Proteolytic enzymatic properties of muscle exudate

Type of enzyme	Substrate	pH	Proteolytic coefficient
Aminopeptidase	dl-leucinamide	7.3	1.6×10^{-4}
Aminopeptidase or carboxypeptidase*	l-leucylglycylglycine	7.6	5×10^{-5}
Aminopeptidase or carboxypeptidase*	dl-leucylglycine	7.6	1×10^{-4}
Aminopeptidase or carboxypeptidase*	dl-alanylglycine	6.6	3×10^{-4}
		7.7	3×10^{-4}
Aminopeptidase or carboxypeptidase*	glycylglycine	5.4	0
		7.5	0
Carboxypeptidase	cbz-glycyl-l-phenylalanine	5.5	0
		7.2	0
Carboxypeptidase	cbz-l-leucylglycylglycine	7.8	0
Pepsinase	cbz-glutamyl-tyrosine	5.2	0
		7.7	0
Trypsinase	benzoyl-l-argininamide	5.5	3×10^{-5}
		8.0	2×10^{-5}

$$\text{Proteolytic coefficient} = \frac{K}{\text{mgm. PN/ml. test solution}}$$

$$K = \frac{1}{\text{time}} \times \log \frac{\text{total moles of substrate}}{\text{total moles of substrate unsplit}}$$

Cysteine was used as an added activator in the experiments run at pH 5 range.

* A recent publication of Smith and Bergmann (84) describes a new type of enzymatic activity termed an imido-endopeptidase which can also hydrolyze these substrates.

of dog muscle exudate on a number of peptide substrates at varying pH's. These substrates have been found (85) to characterize enzymatic activities more precisely than would be possible by means of protein substrates.

The presence of a trypsinase and of an aminoexopeptidase was found. More than 1 enzyme may well be responsible for splitting dl-leucylglycine, dl-leucinamide, l-leucylglycylglycine, and dl-alanylglycine. For the present, however, there is definite evidence of the presence of 1 enzyme which can be designated, for convenience as an aminoexopeptidase. Other observations on this enzymatic activity as it appears in skin, subcutaneous tissue, lymph, and serum have been reported. (86). The possibility was considered that the enzyme might originate from plasma entering the damaged muscle rather than from the muscle substance itself. The level of activity is 5 to 10 times higher in muscle exudate, however, ($C = 5 \times 10^{-3}$) than it is in plasma ($C = 0.6 \times 10^{-3}$), which makes plasma an unlikely origin. Previous mention of the presence of the muscle trypsinase has not reached the authors' attention.

Since it was desirable to compare quantitatively the proteolytic activity found in muscle exudates from different dogs, it was considered best to use the crude muscle exudate as a source of enzyme,

rather than to introduce the possibility of artefacts due to enzyme concentration procedures.

The total amount of proteolytic activity (toward l-leucylglycylglycine) was determined by multiplying the enzymatic activity constant per ml. of muscle exudate by the total volume of muscle exudate. In this way, the total enzymatic activity in various muscle exudates could be compared, and an effort made to see if a correlation existed between the enzymatic activity of a muscle exudate and its toxicity toward an animal.

TABLE III
Negative correlation between toxicity and peptidase activity of muscle exudates

Dog number	Weight	Volume muscle exudate	$K \times 10^{***}$	$V \times K \times 10^3$ kgm. wt.	Result
	kgm.				
147	20	135	20	135	Survived
148	17.5	150	23	197	Died
151	29	280	17	164	Died
153	27.5	400	12	180	Survived
154	23.5	565	8	195	Died
178	7.8	70	28	250	Survived
179	6	90	17	255	Survived
157*	16.3	193	16	195	Survived
144*	5.3	100	45	850	Survived
149**	9.8	50	23	117	Died

* These dogs were given saline extracts of muscle.

** This dog was given heterologous muscle exudate.

*** Substrate l-leucylglycylglycine.

Muscle exudates, therefore, were collected from 7 dogs, and were reinjected in each case intravenously into the same animal from which the fluid was collected. Four of the animals died, and 3 survived. Table III shows that the total amount of proteolytic activity per kgm. of dog reinjected was no greater in the animals which died than in those which survived.

From normal dog muscle, a concentrate of the aminoexopeptidase was prepared which contained 5 times the total amount of aminoexopeptidase found in any of the exudates. Upon injection by slow intravenous drip into 2 normal dogs under sodium pentobarbital anesthesia, no deleterious effect upon the blood pressure, temperature, pulse, or respiration was observed during a subsequent 6-hour period.

The plasma concentration of aminoexopeptidase was followed in 1 dog. It remained constant ($K = 2.1-2.3 \times 10^{-3}$) until after the reinjection of the muscle exudate, and it was found 5 hours after this reinjection to be 4 times its previous level ($K = 8 \times 10^{-3}$).

DISCUSSION

An effort has been made to isolate the toxic factor (or factors) present in certain exudates from anoxic muscle. A study of the electrolyte pattern indicated that potassium and other electrolytes were disturbed but probably not sufficiently to cause shock. Results of dialysis and fractionation with ammonium sulfate pointed toward proteins as containing the toxic substance. Muscle enzymes were suspected, and the proteolytic group of enzymes was investigated. Proteolytic enzymes were demonstrated in the muscle exudates but were found not to be the toxic factor. A saline extract of fresh muscle was found not to be toxic.

These considerations led to the conclusion that some toxic protein component was introduced in-

to certain exudates of anoxic muscle which was not present in extracts of normal muscle. This point of view has directed our attention toward the products of metabolism of the bacteria frequently associated with anoxic, traumatized muscle.

SUMMARY

A study has been made of the fluid which exudes from muscles previously kept anoxic for 5 hours. Some of these exudates produce shock when injected into an animal.

1. There is evidence that this anoxia results in a leakage into the surrounding tissue spaces of intracellular components of the muscle, including proteins and electrolytes. The chemical constitution and electrophoretic patterns of the exudate have been described.

2. The toxic properties of a collection of pooled muscle exudates were contained in the non-dialyzable fraction, could be salted out between 0.25 and 0.7 saturation with ammonium sulfate, and therefore, are probably protein in nature.

3. At least 2 proteolytic enzymes have been found in muscle exudates. One of these, tentatively classified as an aminoexopeptidase, is present in high concentration. This same enzymatic activity can be found in extracts of normal dog muscles. When given intravenously to dogs, a concentrate of this enzyme produced no marked deleterious effect, and the shock-producing effects of individual muscle exudates did not correlate with their peptidase content. The second enzyme found in muscle exudate hydrolyzes benzoyl-L-argininamide, and may be termed a trypsinase.

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Bibliography follows Paper VI of this series.

THE TOXIC FACTORS IN EXPERIMENTAL TRAUMATIC SHOCK. VI. THE TOXIC INFLUENCE OF THE BACTERIAL FLORA, PARTICULARLY *CLOSTRIDIUM WELCHII*, IN EXU- DATES OF ISCHEMIC MUSCLE¹

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As has been shown in previous reports from this laboratory (10, 25, 75), toxic effects resembling traumatic shock have been observed following the intravenous injection of fluids exuding from dog muscles after prolonged ischemia.

The fact that 28 per cent of these fluids were highly toxic, whereas the remainder seemed harmless, strongly suggested that their toxic effects were not due to the presence of intracellular substances lost as the result of cell damage (70). For the same reason, it appeared doubtful that the occasional toxic effects were caused by breakdown products of muscle cell constituents resulting from ischemia, since the experimental conditions were all approximately identical. Attention has been directed, therefore, to the one constituent of these fluids which was obviously highly variable, namely, the bacterial flora.

Another bit of indirect evidence pointed in this direction. On several occasions, dog muscle tissue was excised and minced with sterile precautions and incubated in sterile dog plasma at 37° C., for 5 hours. The plasma was then separated by centrifugation and injected intravenously into recipient dogs. Such material was invariably toxic, rapidly producing a shock-like state and death, and it was always infected with micro-organisms which on direct smear were seen to be large gram-positive rods. However, if a mixture of blended dog muscle and plasma were sterilized by passage through a Seitz filter before incubation,

it never exhibited toxic properties when administered to a recipient animal.

EXPERIMENTAL

Our method of muscle ligation and muscle exudate collection has been described (14). All operative procedures were carried out under sodium pentobarbital anesthesia and with the customary aseptic precautions.

Amounts of fluid exuding from the 2 muscle groups in a 5-hour period after release of ligatures varied in these experiments from 2 to 17 ml. per kgm. body weight of the donor dog. The composition of this fluid and its shock-producing effect when administered intravenously have been described in detail (70, 75).

Practically all of the muscle exudates contained bacteria as demonstrated by direct gram stain. The amount of bacterial contamination in most of these fluids was determined by quantitative culture methods. Five-tenths ml. of the fluid to be tested were mixed with 4.5 ml. of beef broth, and 3 successive 1:10 dilutions in broth were made. Two blood agar plates were then divided into quadrants and a loopful (approximately 1/μl.) of each dilution was streaked onto 1 quadrant of each plate. One of these was incubated aerobically and the other in an atmosphere of 5 per cent CO₂ in nitrogen. After 36 hours of incubation at 37° C., differential colony counts were made and identification of the bacterial species present was undertaken. Colony counts were multiplied by the appropriate factors to give numbers of viable bacteria per ml. of original fluid, and these were checked qualitatively with observations on direct smears. Clostridia were isolated and cultivated from various fluids.

In an attempt to reproduce at will the picture of toxicity following fluid injection, 2 strains of *Clostridia perfringens* thus isolated were used in 3 experiments. In these experiments, at the time of muscle ligation, 2 to 5 ml. of a broth culture of these organisms were injected into each of the 2 muscle groups; after the period of occlusion of the blood supply the ligatures were released as usual and fluid collected. These have been termed "reinforced" fluids. Bacterial counts were made on the first fluid obtained from each leg, on a final sample drain-

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ing off at the end of a 6-hour period, and from the pooled fluid.

A series of dog muscle biopsies have been done in an effort to discover whether clostridia can be grown from samples of dog muscle obtained under aseptic precautions. After preparing the skin in the same way as described for the operative ligation of muscles, the skin was incised and the edges retracted. The subcutaneous fascia was then incised and retracted with a fresh set of sterile instruments, and samples of muscle (about 1 gram) were excised, using a third set of instruments. In other cases, the skin was seared either by a hot scalpel or electrocautery. The remainder of the procedure was accomplished by means of electro-surgical technique. Samples of rectus and sartorius muscle were taken by both these methods. Biopsies of skin were also removed after the usual preparation including a very strong solution of iodine. All biopsy specimens were placed at once into meat infusion broth and incubated in an atmosphere of 5 per cent CO₂ in nitrogen.

RESULTS

In spite of the aseptic precautions, the fluid exudates collected after muscle ischemia have all contained bacteria. In most wounds, the body takes care of such contaminations, but traumatized anoxic tissues offer excellent media for growth and toxin formation. It appears, therefore, likely that traumatizing experiments on dogs must in general be complicated by contamination with bacteria which, in most observations on shock, would have time to exert a toxic influence.

Organisms cultured from fluids. Aerobic cultures were made in 30 instances. One of these cultures was made from a pool of 2 fluids used for injection and another from a pool of 9 fluids, so that 39 exudates are represented. Anaerobic cultures were made of 19 separate fluids, all of which are also represented in the figures given for aerobic flora. Table I shows the results of these cultures, and it will be noted that staphylo-

cocci and clostridia are the most common contaminants, each having been cultivated from the great majority of fluids. Where clostridia were cultured to determine type, as well as morphology, *Cl. perfringens* was obtained.

Bacteria in relation to toxicity. On 19 separate fluids, quantitative bacterial counts were made by both aerobic and anaerobic culture, and the toxicity of 18 of these fluids was determined by intravenous infusion into recipient dogs. In 5 cases, 2 fluids were pooled for assay purposes.

TABLE II
Muscle exudates: correlation of properties with number of Clostridia

Donor dog number	10 ⁵ X Clostr./ml.	10 ⁵ X Staph./ml.	Fluid output ml./kgm.	Recipient dog number *	Toxicity
166	0.001	1.0	7	167	0
217-218	0.01	2.8-3.0	8-6	219	0
221-222	0.03	0-0	5-4	223, 224	0
226-227	0.14	0.06-0	5-3	228, 233	0
214-215	0.17	0.09-0	3-10	216	0
186-187	2.7	0.5-6.0	15-6	188	0
177	2.8	0.8	14	179	0
168	3.0	0.6	17	169	0
181	5.0	2.4	16	183	+
171	35.0	+**	17	174, 175	0
176	160.0	28	8	178	0
180	500.0	40	16	182	+
172	750.0	+**	13	173	+

* The average fluid output of this series of animals is approximately 9 ml. per kgm. In a larger series the average output was 12 ml. per kgm.

** Staphylococci present but not quantitated.

Table II shows the results. It can be seen that the fluids with relatively low bacterial counts were always non-toxic; the 3 toxic fluids were included in the 5 highest clostridial counts. Staphylococcal counts correlated slightly less well with toxicity, but there was considerable correlation between the 2 organisms. There also appears to be some correlation between the bacterial counts and the amounts of fluid produced.

Growth and toxin production "in vivo." In 7 instances, data are available showing the increase in bacterial content of fluid obtained during the 6-hour period after release of ligation. Cultures and colony counts were made on the first fluid and again on fluid accumulating at the end of the period. The results, as shown in Table III, demonstrate the magnitude of the increase. Since many of the initial counts were too low to be established quantitatively under the conditions

TABLE I
Micro-organisms identified in muscle exudates

Organism	Number of times identified	Number of cultures made	Percentage of fluids present
Clostridia	13	19	72
Staph. albus	23	30	78
Staph. aureus	8	30	27
B. subtilis	9	30	30
Coliform bacilli	7	30	23
M. tetragens	4	30	13
Streptococci	2	30	7
Unidentified	2	30	7

used (*i.e.* below 10,000 per ml.), they have been taken as 10^4 per ml. for the purpose of obtaining a figure representing the average of the increments. The calculated mean increment is, therefore, a minimum value. It is seen that there is greater than 1000-fold increase in the concentration of viable clostridia and staphylococci during the 6-hour period following release of the constricting band. Assuming a generation time of approximately 30 minutes, this rapid increase indicates that multiplication of clostridia must have started immediately, as soon as ischemia provided satisfactory anaerobic conditions for growth.

TABLE III

Increase of bacterial counts from initial to terminal fluids

Dog	Clostridia		Staphylococci	
	Initial	Terminal	Initial	Terminal
176	$<10^4$	1.6×10^8	$<10^4$	2.8×10^7
177	4×10^4	2.8×10^8	$<10^4$	2.5×10^8
180	2.5×10^5	1.0×10^9	2.2×10^5	1.0×10^8
181	$<10^4$	3.0×10^7	$<10^4$	1.0×10^7
186			6×10^4	2.5×10^8
187			$<10^4$	2.5×10^7
222	10^4	6×10^4		
- Average	6.4×10^4	2.4×10^8		
Factor of difference:				
(By average of increments)		4×10^3	11×10^2	
(By increment of averages)		4×10^3	5×10^2	

Reinforced fluids. In the case of fluids from muscles which, at the time of ligation, received injections of cultures of bacteria (*Cl. perfringens*) isolated from earlier experiments, extremely toxic exudates were obtained in comparison with those collected in routine experiments. The 3 fluids in this series, when injected intravenously, uniformly produced a shock-like picture terminating in death in 8 instances. In these cases, 5 ml. per kgm. (or approximately one-third of the dose used in the earlier experiments) were given intravenously, and in all cases marked hemolysis was observed in the bloods of the recipients at the time of death. In 3 of the 8 experiments, bacteria had been removed by filtration through a Chamberland candle in 1 case and by centrifugation in the other 2. Washed bacteria obtained by centrifugation were used in 2 experiments and produced no obvious effect except

hyperthermia (41° and 42° C., respectively). In 1 further experiment, a lethal amount of reinforced fluid was injected along with 5 ml. polyvalent gas gangrene antitoxin (Lederle's) with complete protection against the effects of the fluid. Thus, the effects of the reinforced fluids are due to the presence of an exotoxin which is neutralized by gas gangrene antitoxin.

Increases in bacterial content and toxin concentration during the collection period were noted. Tests were made on the first fluid, the last fluid obtained at the end of 6 hours, and the pool of the total fluid obtained. In the case of 1 of these experiments, in addition to bacterial counts, toxicity was determined as the volume of fluid containing 1 mouse m.l.d. There was a 300-fold increase in bacterial count and at least a 10-fold increase in the toxin concentration during the 6 hours after release of ligatures. When a 10 ml. culture was injected into the muscles at the time of ligation, the injected culture contained in all 100 mouse m.l.d. (L.D. 100) of toxin; following release of the muscle ligature, 270 ml. of fluid exuded from the muscle containing 1350 m.l.d. of toxin. It may be assumed that additional toxin was present in the muscles at the end of this period. Thus, the anoxic muscle *in vivo* has been shown to be a good medium for multiplication and toxin production by toxigenic clostridia.

Anaerobic cultures of biopsy specimens. In 11 of 13 intact dogs from which muscle biopsies were obtained, clostridia were grown from at least 1 muscle specimen, and they were cultivated in 13 instances out of a total of 25 such specimens (0.5 to 1 gram of tissue). Thus, clostridia were obtained from 85 per cent of the dogs whose muscles were biopsied. In addition, 36 per cent of the muscle specimens yielded coliform organisms and 8 per cent of them staphylococci.

In 2 dogs, all of 5 cultures of surgically prepared skin were positive for clostridia, and 1 from each dog showed the presence of staphylococci, coliform organisms, and diphtheroids. These data suggest that organisms of the gas gangrene group are common inhabitants of dog skin, where they presumably exist as spores, and are exceedingly readily introduced into the underlying tissues during operative procedures. The possibility that they are normal inhabitants of dog muscle in some instances is not ruled out.

Anaerobic cultures were also made of muscle specimens obtained from 7 rats; 1 of these cultures yielded clostridia, another yielded a culture of gram-positive cocci, and the remainder were sterile.

Eighteen cultures were made of specimens of human muscle obtained from various elective operative procedures.¹ In no case were clostridia grown from these specimens although staphylococci were obtained in 10 cases.

Identity of organisms. Clostridia obtained from muscle exudates and from dog biopsy specimens were identified as *Cl. perfringens* in the following way.

Anaerobic cultures (in broth and on agar media) of muscle extracts and from dog biopsy specimens yielded large numbers of organisms which were identified as *Cl. welchii* (*perfringens*) on the following grounds.

The colonies and broth cultures were found to consist of rods approximately 1μ in width and varying from 3 to 10μ in length. Very young organisms were frankly gram-positive but became gram-negative within 24 to 36 hours, all intermediate degrees of intensity of staining being observed in 1 given culture according to its age.

Spores were very rarely encountered and in particular could not be detected in sugar media.

The stormy fermentation of litmus milk and other fermentation reactions were typical of *Cl. welchii*. Gelatin was liquified but no digestion of serum was observed.

The colonies were either smooth or rough, hemolytic or non-hemolytic (β hemolysis). The fact that these different colonial types could all be derived from 1 single colony isolated upon aging of the culture indicates that they were due to bacterial dissociation rather than being representatives of different bacterial species.

The cultures grown in ordinary glucose broth were only a little toxic for mice (MLD 0.1 ml.). Toxicity could be much increased by growing the organism in meat media containing 0.5 per cent glucose or in media specially devised for the production of Welch toxin (MLD 0.001 ml.).² The toxin could be neutralized by Welch antitoxin.

¹ We are indebted to Dr. Carroll B. Larson for the bulk of these specimens.

² This toxin was supplied by Dr. A. Pappenheimer, Jr.

DISCUSSION

The presence of bacteria of the gas gangrene group in normal dog muscle has been reported (87, 88), and both our studies and the confirmatory one of Cope and Langohr (unpublished data) have indicated that such organisms may be normal inhabitants of dog muscle; but it is more likely that they are introduced during operative manipulations, since they appear to be such frequent contaminants of dog skin. In all probability, they exist in skin as spores resistant to the usual methods of surgical preparation. In the dog, therefore, gas bacilli are potential contaminants of artificially-traumatized or surgically-treated tissues, and all experimental procedures which tend to result in ischemia and anoxia would produce conditions extremely favorable for their multiplication and toxin formation.

It should be emphasized, also, that from both biopsy material and "sterile" collected exudates from ischemic muscles, other microorganisms than clostridia, especially staphylococci and bacilli of the coliform group, have been frequently cultivated. The creation of damaged and relatively ischemic tissue tends to promote the growth of many types of pathogens, especially anaerobes, and among the commonly contaminating organisms are included several species capable of producing powerful exotoxins (89, 90). The ready accessibility of clostridia, the fact that conditions for their growth and toxin production are optimal, and the potency of their characteristic toxins make it important to investigate the rôle which such bacteria might play in the sequence of changes that constitute the syndrome of traumatic shock.

The toxins of several species of bacteria have been shown to have specific pharmacologic effects on the circulation with certain features in common with the classical changes of shock. Notable examples of these are the exotoxins of gram-negative bacilli (89, 90). The production of hemolysis and death in rabbits by the intravenous administration of Welch bacillus toxin has been reported (91), and the action of this toxin has been further described (92). Experiments in this laboratory with purified *Cl. perfringens* and *Cl. oedematiens* toxins have confirmed these results in dogs and demonstrated both local and generalized toxic effects of these agents on the circulatory system.

These facts make it clear that the element of bacterial infection and its consequences are factors that must be taken into consideration in physiologic experiments which continue for longer than a few hours. Particularly in experiments on traumatic shock in which large areas of damaged and ischemic tissue are produced, the rôle of bacterial products in contributing towards the clinical outcome cannot be ignored. Interpretation of the results of experiments in which parenteral administration of various extracts of normal and traumatized tissues is made should involve consideration of the bacterial factor (93). This has been recognized and it has been shown that bacteria-free extracts of these types are without physiologic effects (65).

The possible rôle of micro-organisms in the genesis of traumatic shock following the injury of men in battle remains to be evaluated. Although human muscle biopsy specimens have been, in our experience, generally sterile, wounds sustained under battle conditions are usually contaminated with many species of bacteria, and the gas bacillus ranks high in its incidence in war wounds (94, 95). Certainly in many of the latter, conditions must be very favorable for the multiplication of such contaminants, and it is conceivable that sub-clinical amounts of highly toxigenic strains of bacteria, especially of the gas gangrene group, might produce sufficient quantities of toxin to be a factor in the development of circulatory failure, either by promoting loss of vascular fluid at the site of trauma or by acting generally on the cardio-vascular system. That the former may be a not insignificant effect in the case of the Welch bacillus toxin has been suggested (96). The irreversibility of the shock-like state produced by the intravenous administration of *Cl. perfringens* and *Cl. oedematiens* toxins (unpublished data) indicates that the production of toxins of these types in contaminated wounds can play a significant rôle in subsequent development of "irreversible" shock.

These considerations and especially the urgency of elucidating the pathogenesis of irreversible peripheral circulatory failure make it important to study human cases of traumatic shock from the point of view of the bacteriology of their wounds in order to determine what counterpart the findings reported in this paper have in clinical shock.

SUMMARY

The bacterial flora of ischemic canine muscle exudates has been investigated. *Clostridium perfringens* and *Staphylococcus albus* have been recovered from most of these exudates in widely varying concentrations. The clostridia have been shown to multiply and produce toxin in ischemic muscle, the multiplication starting as soon as ischemia provided satisfactory anaerobic conditions for growth. They are present in many biopsy specimens of normal dog muscle obtained with the use of rigid surgical techniques of skin sterilization and sterile handling of specimens. Clostridia are not present in normal human muscle.

Evidence has been obtained indicating a correlation between the toxicity of the fluids as administered to recipient dogs and their bacterial content. We think this bacterial contamination is the source of a "toxic factor" in experimental shock. Bacterial infection and its consequences must be taken into consideration in physiological experiments, particularly in those involving injury to tissue, even where performed by surgical methods.

The possible relation of clostridial infection to human wound shock remains to be elucidated. The frequent contamination of war wounds with these organisms indicates the importance of such an evaluation.

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SOME OBSERVATIONS ON THE EFFECT OF INJECTED CYTOCHROME C IN ANIMALS¹

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In previous communications it was suggested that respiratory catalysts might be used to help maintain normal functions of tissues under conditions of anoxia by their enhancing effect on tissue respiration (1, 2, 3, 4). Even under conditions of anoxia, there is in the returning venous blood a considerable amount of oxygen which might theoretically be made available to the anoxic tissue through the effect of an additional supply of a respiratory catalyst. That one could apparently produce an increased local tissue uptake of oxygen under conditions of anoxia *in vivo* through the use of the hydrogen donator, succinic acid, has previously been demonstrated (1, 2). Cytochrome C, in combination with cytochrome oxidase, is one of the most important respiratory enzyme systems and the following report deals with some effects of cytochrome C. An additional reason for the selection of cytochrome C for our studies is the fact that it is present in normal tissues in amounts far below what is required for the full activation of the cytochrome-cytochrome oxidase system. That is to say, there is relatively more cytochrome oxidase present in the organs than is necessary for activation by the cytochrome C present, as noted in Table I. These figures are

TABLE I

Gammas cytochrome C per gram of fresh tissue

Organ (rat)	Required for half of maximal activation of oxidase	Found by analysis
Heart	1700	371
Kidney	860	247
Liver	675	90
Brain	720	50

based on calculations from data reported by other workers (5). Hence, if additional cytochrome C could be supplied to the organs, it might be expected to be effective.

¹ This work was done with the aid of grants from Brewer & Company, Inc., and the Charlton Fund.

The accumulation of injected cytochrome C in organs

It is obvious that injected cytochrome C can be effective only if it actually penetrates to the organs. Since cytochrome C is a protein and thus a large molecule, there was uncertainty as to whether it would pass through the capillaries sufficiently to accumulate in the organs in effective amounts.

TABLE II

Cytochrome C content of organs and blood of rats before and after injection

1. Cytochrome C content of organs (gammas per gram) of rats

No. of animals used	Organ	Minimum	Maximum	Average
15	Heart	185	270	220
16	Kidney	126	245	188
15	Liver	60	165	120
2	Spleen	105	110	108

2. Cytochrome C content of organs (gammas per gram) and blood (gammas per 100 ml.) of rats 30 minutes after intravenous injection of Cytochrome C

Amt. of Cyt. C injected	1 mgm.	2 mgm.	5 mgm.	10 mgm.	20 mgm.	50 mgm.
Blood		35	102	185		257
Heart	393	305	440	230		215
Kidney	360	362	291	350		765
Liver	163	187	225	250		178
Spleen	140	290	147	570	680	

Furthermore, if it did accumulate, it was necessary to obtain information as to how long it remained unchanged in the organs. We found that it was in fact possible to increase the organ content of cytochrome C after injection and that this injected substance was relatively stable.²

Table II shows the amounts of cytochrome C in organs and blood of control animals (rats) and in

² The method of determining the organ content of cytochrome C was that of Potter and Dubois (5). The method for preparing cytochrome C was that of Keilin and Hartree (6).

animals following the intravenous injection of varying amounts of cytochrome C. It will be noted that there is a distinct increase in the organ content of cytochrome C following the injection of as little as 1 mgm. of the substance. It is of interest to note further that increasing amounts of injected cytochrome C beyond a certain amount (in these experiments, 5 to 10 mgm. intravenously) seem to produce a paradoxical effect on the content of cytochrome in the heart and liver so that with doses of more than 5 to 10 mgm. of cytochrome C intravenously, there is not a further increase in these organs of cytochrome C, but rather a decrease toward the normal values. On the other hand, in the kidneys and spleen and in the blood as well the content of cytochrome C continues to rise with increasing doses of the injected substance.

TABLE III

Cytochrome C content in organs of rats after injection intravenously and intramuscularly

1. Cytochrome C content in organs of rats (gammas per gram) after intravenous injection of 2 mgm. of cytochrome C				
Time after injection	minutes		hours	
	30	60	2	15
Heart	378	303	303	165
Kidney	380	335	150	120
Liver	203	142	76	58

2. Cytochrome C content in organs of rats (gammas per gram) after intramuscular injection of 2 mgm. of cytochrome C				
Time after injection	minutes		hours	
	30	60	2	15
Heart	261	234	360	220
Kidney	203	222	240	254
Liver	113	108	124	175

Table III shows the relative increase in the cytochrome C content of organs following intramuscular and intravenous injections. There is seen to be a slower and less marked rise following intramuscular injection as compared with the intravenous injection.

Changes similar to these in rats were observed after injections of cytochrome C in rabbits.

Physiological effects of injected cytochrome C.

In vitro experiments. Having demonstrated that cytochrome C is relatively stable in the intact

animal and that the organ content of the substance could be increased following parenteral injection, it remained to determine whether such an increase of cytochrome C as we could produce in the organs was of a sufficient magnitude to increase the oxygen uptake of the tissue. We approached this problem by studying the influence of added cytochrome C on the oxygen uptake of isolated rat organs. That the increase of cytochrome C of this general order of magnitude could produce a significant increase in the oxygen uptake of homogenized tissue suspensions is demonstrated in Table IV. The concentration of cytochrome C in

TABLE IV

O₂ consumption during 10 minutes incubation at 37° C. (mm.³ O₂ per 100 mgm. fresh tissue)²

Organ (rat)	No cytochrome C added beyond usual content	300 gammas cytochrome C added per ml. medium
Liver	41	60
Kidney	50	79
Kidney	54	97
Heart	24	50

the Ringer's solution serving as medium for the homogenized tissue suspensions was made to correspond with that found in the organs of the injected animals.

It will be seen that there is under such circumstances an increase of oxygen consumption of about 50 to 100 per cent. It would appear, therefore, on theoretical grounds that we were able to increase significantly the conditions for oxygen uptake in the organs studied by the increase in the cytochrome C content of the organs resulting from the injections.

In vivo experiments. The observations on the enhancing effect of added cytochrome C on the oxygen uptake of isolated tissues raised the question as to whether similar effects might be demonstrated in intact animals. The total oxygen consumption of normal dogs was found to be unchanged after the injection of as much as 50 mgm. of cytochrome C intravenously. We then made observations on the arteriovenous oxygen differences to determine whether there was evidence of increased tissue uptake of oxygen as indicated by a decrease in the venous content of oxygen. The venous blood was obtained under normal condi-

² Total volume 35 ml., pH 7.4, 0.5% (v/v) glucose, 0.5% (v/v) bovine serum albumin, 0.5% (v/v) NaCl, 0.5% (v/v) platelet mixture (pH 7.2).

C under conditions of anesthesia and anoxia. The answer appears to be that there is a reciprocal relationship between the cytochrome C content of the blood and the liver, so that as the level in the blood decreases as a result of anoxia, the content in the liver simultaneously increases. This reciprocal relationship is demonstrated in Figure 4. Our observations on this phenomenon were limited to the liver because this is the only organ easily susceptible to repeated biopsies for tissue analyses without serious impairment to the total functioning of the body. A similar situation may well be obtained in the other organs. This phenomenon suggests that the supply of cytochrome C circulating in the blood stream acts as a reservoir to be called upon as the need arises and to be replenished when the need no longer exists.

The ultimate fate of the injected cytochrome C is obscure. There is reason to suspect that it is at least largely broken down before excretion inasmuch as we were never able to recover it in the urine. Nor were we able to recover porphyrin, one of its possible breakdown products.

SUMMARY

1. The cytochrome C content of organs (heart, brain, liver, and kidney) is far below that required for the maximal activity of the cytochrome oxidase present. This apparent suboptimal condition for the action of the cytochrome-cytochrome oxidase system in normal tissues led us to attempt first to increase the cytochrome C content of the organs and then to study the effects resulting from supplying such additional cytochrome C.

2. We find that cytochrome C prepared from beef hearts is stable, non-toxic in large doses, recoverable from the blood, and in increased amounts from the organs after intravenous and intramuscular injections, and apparently broken down before excretion because it is not recoverable in the urine.

3. Following the intravenous injection of cytochrome C, there is a considerable increase in the content of this substance in the heart, liver, and kidney of the rat and rabbit. There is a similar, though delayed, increase following intramuscular injection.

4. It has been demonstrated by *in vitro* experiments that an increase of cytochrome C, similar to that produced in living organs by injection is sufficient to produce a significant increase in tissue uptake of oxygen.

5. In the dog, under conditions of anoxia, injected cytochrome C increases the arteriovenous oxygen difference, presumably by increasing the withdrawal of oxygen from the arterial blood. Under conditions of normal oxygen tension, injected cytochrome C does not influence the arteriovenous oxygen difference in man.

6. By daily intravenous injections, a satisfactory blood level can be maintained.

7. Under conditions of anoxia, the liver content of cytochrome C increases after injection, whereas the blood level decreases. With release from anoxia, there is a decrease in liver content and an increase in blood level. This suggests that the supply of cytochrome C circulating in the blood stream acts as a reservoir to be called upon as the need arises and to be replenished when the need no longer exists.

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EFFECT OF IODINE ON THE THYROID GLAND IN GRAVES' DISEASE WHEN GIVEN IN CONJUNCTION WITH THIOURACIL— A TWO-ACTION THEORY OF IODINE¹

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The improvement observed in patients with Graves' disease following the administration of iodine is a phenomenon which has excited considerable speculation as to its mechanism.

Plummer (1) who introduced (or better, re-introduced) iodine in the therapy of Graves' disease, advanced a 2-product theory of thyroid secretion in that malady. His suggestion was that an incompletely iodinated hormone is secreted in Graves' disease which has toxic properties not possessed by the normal hormone. The improvement under iodine therapy he attributed to restoration of complete iodination of the hormone molecule.

In this clinic, studies have been in progress on the action of iodine in Graves' disease ever since Plummer's contribution in 1923. For his 2-product theory, there has been found little if any convincing support, but his claims as to the benefit conferred by iodine in Graves' disease have been abundantly confirmed.

In contrast to Plummer's 2-product theory, we wish to suggest a theory that iodine has 2 quite distinct actions upon the thyroid, not only in Graves' disease, but perhaps under other circumstances as well.

The germ of a 2-action theory of iodine goes back a good many years in our clinic. For example, in 1929 (2), the following opinion was expressed: "We believe that for the present, at least, it will be wholesome for the profession to look upon the action of minute amounts of iodine in preventing endemic goiter as being in its nature very different from that of far larger quantities in bringing about detoxication in exophthalmic goiter."

The primordial rôle of iodine in thyroid economy is obviously that of supplying an ingredient essential to hormone synthesis. We may call this the "iodinating action." It has been estimated that in the healthy human this function can be served by as little as 0.075 mgm. of iodine per day. If an excess of iodine, that is to say, more than is necessary to serve the iodinating function, is administered to a normal person, it exerts no noteworthy effect. The thyroid traps the iodine needed and lets the rest go by to be excreted. If the quantity of iodine ingested is insufficient to fill the needs of the iodinating function, an iodine-want type of goiter develops.

The picture is very different in Graves' disease. Here the thyroid is rapidly utilizing available iodine in the manufacture of thyroid hormone and has an avidity for iodine which is about 2 to 3 times that of the normal human thyroid. In this disease when iodine is administered in adequate amounts involution of the thyroid and detoxication of the patient results. However, the dose of iodine necessary to produce this effect is 6.0 mgm. or more daily (3), an amount that is at least 80 times as great as the amount of iodine necessary to prevent the development of an iodine-want goiter. This action of iodine we can call the involuting action.

The manner in which this involuting action is produced has been obscure. Because of the similarity of the curves of the metabolic response to iodine in Graves' disease and of the thyroxine decay in the thyroidectomized subject, it was concluded that in Graves' disease the effect of a relatively large supply of iodine is to block the delivery of thyroid hormone from gland to body (4). Other workers have reached similar conclusions. Such an interruption of hormone output could be due to a saturation of the hormone

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facture or to some barrier to the escape of hormone from the gland. Certain observations (5) would support the latter. It was demonstrated that the total and thyroxine-like iodine of thyroids removed from thyrotoxic patients after iodine treatment were as great or greater than those observed in normal human thyroids in contrast to the low values observed in the thyroids of thyrotoxic patients who had had no iodine therapy.

The theory was advanced (6) that at least part of the action of iodine was to suppress the secretion of the thyroid-stimulating hormone of the pituitary. It was proposed that part of the action of iodine was to prevent the action of the thyrotropic hormone (7).

It has been suggested (8) that "the effect of iodine therapy is to favor the synthesis and deposition of thyroid hormone rather than its release." More recently this theory has been elaborated upon by an investigator who considers this the best explanation as to why iodine is beneficial in the treatment of Graves' disease (9). He suggests "that the gland is made to secrete internally into its follicles rather than externally into the blood stream. Thus iodide in excess," he suggests, "may be said to reverse the direction of flow of hormone."

In the light of the theory advanced by these investigators (8) it can be argued that what we have defined as separate actions of iodine are actually no more than 2 aspects of a single action. Such an argument can be refuted by separating the 2 actions. Such a separation involves the use of a goitrogenic drug like thiouracil. It has been demonstrated (10 to 13) that under sufficiently large and sustained dosage with this drug, utilization of iodine for hormone manufacture by the thyroid can be kept in abeyance. Under this treatment, the thyroid becomes hyperplastic due to pituitary stimulation, but still no thyroid hormone is synthesized because of the impediment to iodination exerted by the goitrogenic drug. Yet, if in Graves' disease while continuing the suppression of the iodinating action through the administration of thiouracil, iodine is given in addition, as the following observations demonstrate, involution of the thyroid can be induced. Thus, under these circumstances the iodinating action and the involuting action have been divorced.

OBSERVATIONS, MATERIAL AND METHODS

For the purpose of separating the iodinating and involuting actions of iodine, the following studies were made in 7 patients suffering from Graves' disease. In 4 patients the histology of the thyroid was studied in sections of biopsy specimens taken before any therapy, and also after a fall in the rate of metabolism to a standard level had been produced by the administration of thiouracil. These histologic specimens were compared with sections taken from several areas of the operatively-removed thyroids after continued thiouracil and added iodine treatment. In 3 other patients the histology of 1 biopsy specimen, taken from the thyroid after treatment with thiouracil had produced a fall in the rate of metabolism to a standard level, was compared with the microscopic picture of several histologic specimens taken from the operatively-removed thyroid after iodine had been added to the continued thiouracil treatment.

The urinary excretion of tracer doses of radioactive iodine was determined in 3 cases before beginning any form of treatment and in 6 cases after thiouracil treatment had caused a fall in the rate of metabolism to a standard level. Finally, the total iodine and the thyroglobulin iodine of each operatively-removed thyroid were determined.

All of the iodine administered to 1 patient, A. S., No. 24684, was labelled with radioactive iodine and thus a balance study was made. This patient was a 16-year-old female who suffered from a moderately severe Graves' disease. Her pretreatment basal metabolic rate level was plus 35. Before beginning treatment, she received 150 micrograms of iodine as sodium iodide labelled with 100 microcuries of 8-day half life radioactive iodine, and the urinary excretion of the labelled iodine was followed. Two days later a biopsy was taken from her thyroid. Following the biopsy thiouracil was administered, 0.2 gram every 6 hours. Her basal metabolic rate fell to a level of plus 10 in 8 days. On the eighth day of treatment, a second tracer dose of iodine was given, and another biopsy was taken from her thyroid on the tenth day. Following the second biopsy, thiouracil treatment was continued and 300 mgm. of iodine as sodium iodide were administered daily for 10 days until the thyroid was removed. The iodine was exhibited 1 hour after 1 0.2-gram dose of thiouracil had been given. Each daily dose of iodine was labelled with 100 microcuries of 8-day half life radioactive iodine. All urine was collected and the iodine excreted was determined by measuring the radioactivity in the urine. The radioactivity contained in several specimens of the operatively-removed thyroid was also determined on alkaline digests of these samples.

The patients on whom these studies were made were all females varying in age from 16 to 37 years. All but 1 had a moderately severe thyrotoxicosis with pretreatment BMR levels which ranged between +20 and +55. A. H., No. 25234, was only mildly thyrotoxic and probably not a good subject for this study. It is interesting to note, however, that her thyroid thyroglobulin iodine was quite low (see Table II). Only 2 of the patients, R. M., No.

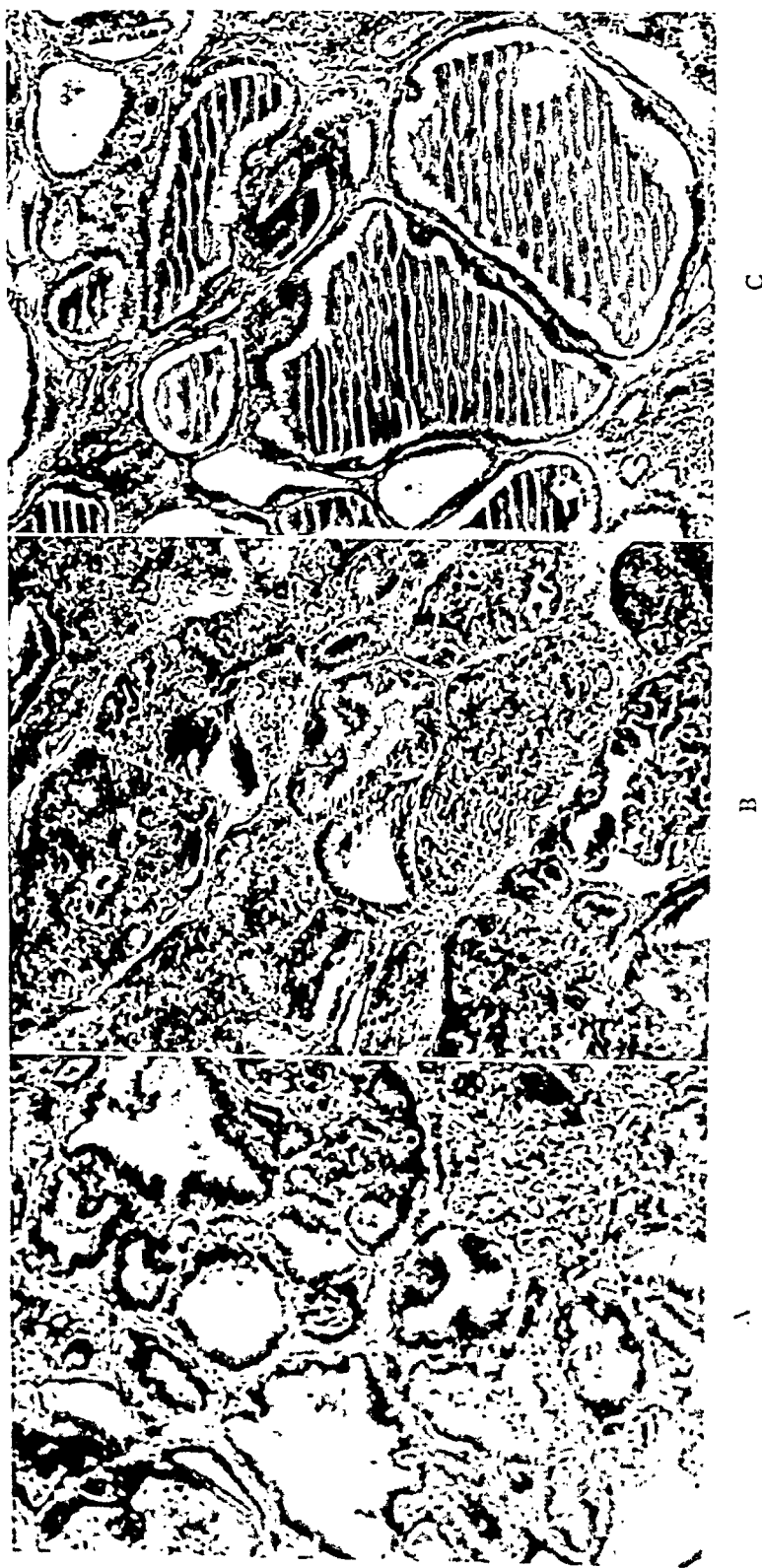


FIG. 1. THYROID GLAND, CASE NO. 25097. MAGNIFICATION = 170 X.
A. Before treatment. B. After response to thiouracil. C. After thiouracil plus iodine.

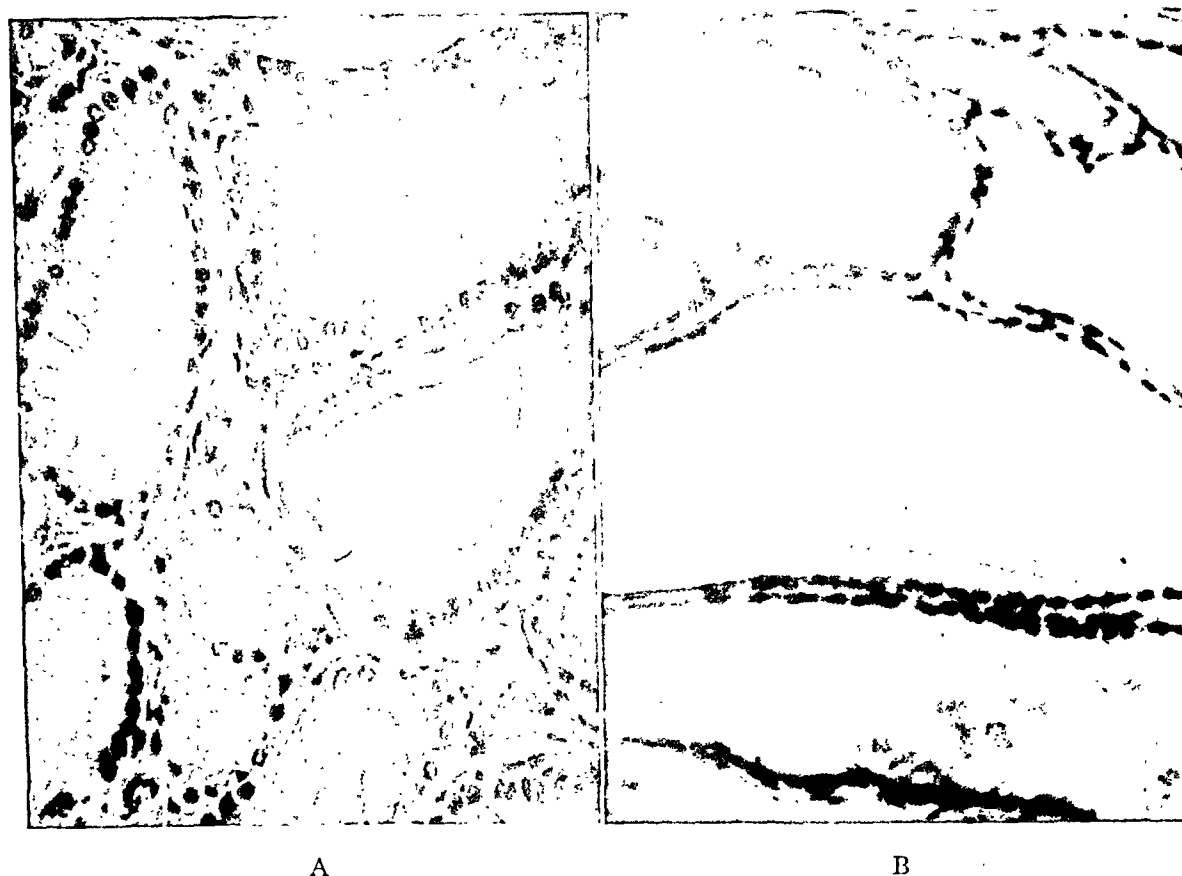


FIG. 2. THYROID GLAND, CASE No. 25314. MAGNIFICATION = 450 X.

A. After response to thiouracil. B. After thiouracil plus iodine treatment.

25314, and E. L., No. 25198, had had iodine in any form within 6 months before thiouracil treatment was begun.

The histologic studies of thyroid biopsy specimens and operatively-removed thyroids were compared quantitatively by determining the mean acinar cell height of each specimen. This was done by measuring under oil immersion the height of 1 representative cell from each of 100 successive acini and then determining the mean of each plotted curve.

The urinary excretion of radioactive iodine was determined by comparing the radioactivity in a measured sample of the urine with a standard taken from the original sample given the patient. The sample of radioactive iodine given the patient contained 100 microcuries of 8-day half life radioactive iodine with 150 micrograms of iodine as sodium iodide. All urine was collected and saved for 48 hours after administering the labelled iodine.

The thyroids after being weighed were minced in a Waring blender and then digested in 0.01 N sodium hydroxide and filtered through gauze. A sample was then removed for determination of total iodine and another sample was removed and dialysed. Thyroglobulin was then precipitated by lowering the pH to 5.4 to 5.6 with slowly added 0.1 N acetic acid. The iodine was determined in each of the 3 samples by the method of Astwood and Bissell (11).

The thiouracil was administered in 0.2 gram doses every 8 or every 6 hours throughout the period of study. Iodine

was administered to all patients except A. S., No. 24684, as saturated potassium iodide 0.3 ml. (330 mgm.) daily. The iodine was given 1 hour after one 0.2-gram dose of thiouracil had been administered.

RESULTS

Mean acinar cell heights of thyroid parenchyma are given in Table I. These show in 4 cases,

TABLE I

Thyroid cell heights, before and during thiouracil treatment, and after thiouracil plus iodine treatment

Patient	Lab. no.	Mean acinar cell height in micra		
		At biopsy before any treatment	At biopsy after thiouracil treatment	At operation after thiouracil plus iodine treatment
C. T.	24972	12.6±0.14*	13.9±0.14*	6.2±0.03*
A. H.	25234	11.1±0.13	10.8±0.13	7.4±0.05
H. M.	25097	13.9±0.19	14.3±0.15	8.2±0.05
A. S.	24684	15.3±0.14	17.3±0.17	10.3±0.07
E. L.	25198		13.8±0.16	6.4±0.01
M. D.	25105		13.8±0.12	6.3±0.05
R. M.	25314		13.5±0.12	5.8±0.05
Average		12.9	13.9	7.2

* Standard error of mean.



Fig. 3. THYROID GLAND, CASE No. 24684. MAGNIFICATION = 170 X.
A Before medication. B, After thiouracil. C, After thiouracil and iodine.

before any treatment was given, an average of 13.2 micra. In the specimens removed at biopsy in these same 4 cases, after thiouracil, the average mean acinar cell height had risen to 14.1 micra. The average mean cell height in 7 cases after thiouracil, but before iodine was 13.9 micra. In these same 7 cases after both drugs had been exhibited the average mean acinar cell height had fallen to 7.2 micra. The histologic changes observed in these thyroids are illustrated in Figures 1 to 3.

In Table II are shown values for the excretion of radioactive (labelled) iodine in 3 cases before any drug was given, and in 6 after treatment with thiouracil. The average excretion before thiouracil was 25 per cent of the administered dose. In these same 3 cases after thiouracil treatment, it had risen to 83.7 per cent. In 6 cases after thiouracil treatment, the average radio iodine excretion was 80.3 per cent. This increased radioactive iodine excretion in the thiouracilized patient strongly suggests diminished utilization in the gland.

TABLE II
*Radioactive iodine excretion before and after
thiouracil treatment*

Patient	Lab. no.	Before treatment	After treatment
		<i>per cent</i>	<i>per cent</i>
C. T.	24972	12.7	94.5
A. H.	25234	46.0	83.2
A. S.	24684	16.3	73.5
R. M.	25314		93.0
E. L.	25198		61.0
M. D.	25101		76.6
Average		25.0	80.3

The iodine content and distribution in the thyroids removed at operation, after both drugs had been exhibited, is given in Table III. These values are calculated as milligrams of iodine per 100 grams of fresh thyroid tissue.

The total iodine values obtained varied between 6.4 and 41.3 with an average of 22.1 mgm. The iodine values of the acid precipitate of digests of these tissues varied between 1.1 and 18.5 and averaged 7.0 mgm. In 5 cases the iodine values of digests of the tissue after dialysis varied between 1.4 and 21.8, averaging 8.3 mgm. The thyroglobulin iodine values here are very low. The significance of these low thyroglobulin iodine

TABLE III
Iodine values of thyroids removed at operation

Patient	Lab. no.	Fresh tissue		
		Total iodine	Thyroglobulin iodine	Non-dialysable iodine
		<i>mgm. per 100 grams</i>	<i>mgm. per 100 grams</i>	<i>mgm. per 100 grams</i>
C. T.	24972	6.4	1.2	
H. M.	25097	10.9	7.4	1.4
A. H.	25234		1.1	
A. S.	24684	24.7	2.3	2.9
E. L.	25198	26.2	6.5	10.7*
M. D.	25105	41.3	18.5	21.8†
R. M.	25314	23.4	12.2	5.0‡
Average		22.1	7.0	8.3

* Had taken iodine for 3 months until 3 months before thiouracil was administered.

† Received 1 dose of iodine when theoretically free of thiouracil effect (see text).

‡ Had taken iodine for 5 months until 1 month before thiouracil was administered.

values becomes more impressive when compared with the values obtained by the same techniques on normal human thyroids, thyroids removed from thyrotoxic patients treated with iodine alone and on thyroids removed from thyrotoxic patients after treatment with thiouracil alone. The total and thyroglobulin iodine values determined on a small series of normal human thyroids averaged 67.2 and 29.8 mgm. per 100 grams of fresh thyroid tissue respectively. The thyroids of another small series of thyrotoxic patients removed after an adequate response to iodine therapy had averaged total and thyroglobulin iodine values of 50.8 and 36.6 mgm. per 100 grams of fresh thyroid tissue respectively. The total thyroid iodine of 4 thyrotoxic patients whose thyroids were removed after thiouracil alone varied between 2.95 and 30.4 and averaged 18.8 mgm. The thyroglobulin iodine values obtained on these same glands ranged between 1.07 and 11.8 and averaged 6.82 mgm. per 100 grams of fresh tissue. The thyroglobulin values obtained on this latter group of patients are comparable to the values obtained in our study of patients after treatment with thiouracil and iodine together.

In comparing the iodine values on these thyroids, it is of interest to point out that patients E. L., No. 25198, and R. M., No. 25314, had received iodine for periods of 3 to 5 months until 3 to 1 months respectively before thiouracil treatment had been begun, and that the somewhat

higher thyroglobulin and non-dialysable iodine levels observed in their glands might be accounted for on that basis. M. D., No. 25105, gave no history of having taken iodine previous to her entry which might account for the elevated total thyroglobulin and non-dialysable iodine levels observed in her thyroid. However, on the day that the biopsy was taken from her thyroid she received 0.2 gram of thiouracil at 6 a.m. and none again until 10 p.m. of the same day. At 6 p.m. of that day she received 330 mgm. of potassium iodide. It has been demonstrated (14) that a 0.2-gram dose of thiouracil is practically all excreted in 8 hours. It has also been demonstrated (13) that the block to the collection of iodine produced by thiouracil lasts only between 6 and 12 hours. It would seem quite likely then that the higher level of thyroid iodine observed in this patient's tissue was due to the administration of iodine when the patient's thyroid was free of any thiouracil effect.

The urinary excretion of the labelled iodine given A. S., No. 24684, is represented graphically in Figure 4. It will be observed that this patient excreted only 16.3 per cent of a tracer dose of

iodine given 48 hours before her pre-treatment biopsy was taken. After she had received thiouracil, 0.8 gram daily for 15 days, she excreted 73.1 per cent of a similar tracer of radioactive iodine. Following the second biopsy taken from her thyroid, all of the iodine which she received was labelled with 100 microcuries of radioactive iodine daily. The radioactivity demonstrated in the excreted urine during and after the 10-day period of treatment with thiouracil and labelled iodine practically equalled the radioactivity administered. Indeed, when it is totalled, 104 per cent of the administered radioactive iodine was demonstrable in the urine. The radioactivity demonstrated in the operatively removed thyroid was reported as being too little to measure.

DISCUSSION

From the observations made in this study it becomes quite apparent that the iodinating action can be separated from the involuting action of iodine on the thyroid, inasmuch as thiouracilized patients exhibit the involuting action without iodination of the hormone.

It has been stated by Harington (15) that "the first event in the synthesis of thyroxine by the thyroid gland must be substitution of the iodine in the molecule of an organic compound and such substitution can only occur after oxidation of the iodide." It has been reported (16) with *in vitro* experiments that thiouracil inhibits peroxidase activity in the thyroid and that this diminished peroxidase activity would interfere with the conversion of diiodotyrosine to thyroxine. Since it has been amply demonstrated in previous studies that thiouracil will prevent the concentration of iodine in the thyroid, and in these studies that it will interfere with the iodination of thyroid protein, it might be suggested that the enzyme which has been found to be inhibited has as a primary function the oxidation of iodide to iodine.

Whether it be peroxidase which has to do with the iodination of the thyroid protein or some other enzyme, the evidence from these observations would indicate that iodine when administered in doses of 300 or more mgm. daily to adequately thiouracilized patients is inutile for the iodination or synthesis of thyroid hormone. However, notwithstanding this barrier to the production and

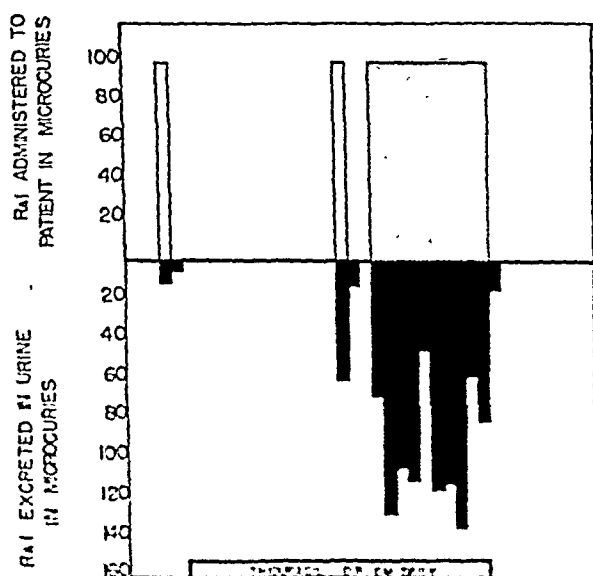


FIG. 4. RADIO-IODINE BALANCE IN CASE No. 24684

Intake above the line. Urinary excretion below. With regard to intake plain columns indicate that tracer iodine was given with carrier of 150 gamma of ordinary sodium iodide. Stippled columns indicate that tracer iodine was given with carrier of 500 mgm. daily of ordinary sodium iodide. The latter carrier amounted to a therapeutic dose of ordinary iodine. (See text.)

storage of a normally iodinated thyroid hormone, the administration of iodine to a patient having Graves' disease causes involution of the hyperplastic thyroid so characteristic of that malady.

These observations make untenable the theory (1) that iodine causes detoxication of the patient with Graves' disease by bringing about complete iodination of the hormone molecule. Likewise, the theory (8) that "the effect of iodine therapy is to favor the synthesis and deposition of hormone rather than its release" will require modification. It is true that the administration of iodine to a thyrotoxic patient will promote the storage of thyroid hormone. However as our observations demonstrate, involution of the thyroid is not dependent upon the deposition of hormone. A more tenable explanation would be that the storage of thyroglobulin results from the inhibition of some process which has to do with the increased rate of secretion of thyroid hormone. It has been demonstrated (17) that the 2 earliest effects on the thyroid produced by the administration of thyrotropic hormone are an increase in the mean acinar cell height and a loss of stored iodine. An increase in the rate of collection of iodine was not observed until later. Removal of thyrotropic stimulation or inhibition of this hormone's action should result then in involution of the hypertrophied thyroid cell and in a cessation of increased secretion of hormone. The apparent increased deposition of thyroid hormone could then be due to a block in the delivery of hormone (4).

In 1941, it was reported that explants of thyroid tissue, when bathed in a medium containing pituitary extract, inactivate the thyrotropic hormone, whereas explants of other tissues, excepting thymus and lymph nodes, have no effect on the thyrotropic hormone exposed to them (18). On the basis of these observations, we have thought that the thyrotropic hormone in exerting its action on its endorgan is inactivated possibly by combining with some substance in the thyroid or by contributing some essential part of its molecule to the metabolism of the thyroid cell. In another series (19) of *in vitro* studies, we have observed that the inactivation of thyrotropic hormone contained in the bathing medium upon exposure to explants of thyroid tissue slices was almost completely inhibited if iodide was contained in the medium. These observations would indicate then that iodine

exerts its involuting effect in Graves' disease by inhibiting the action of the thyrotropic hormone.

A major criticism to the theory that the involuting action of iodine is brought about by inhibiting the action of thyrotropic hormone on the thyroid is that iodine, when administered to normal individuals, has not been observed to cause any clinical hypothyroidism. Indeed, its administration to patients with Graves' disease only rarely causes a state of hypothyroidism.

On the other hand, it might be pointed out that in another *in vitro* study it was observed (20) that explants of tissue slices taken from thyroids of patients with Graves' disease inactivated about twice as much thyrotropic hormone as did similar explanted slices taken from normal human thyroids. It may be suggested then that iodine, when administered to patients with Graves' disease, acts by inhibiting an increased sensitivity of the thyroid cell to the action of thyrotropic hormone and that since this increased sensitivity to thyrotropic hormone does not exist in normal human thyroids, the action of iodine in these people does not cause any dramatic effect.

SUMMARY

Patients with Graves' disease have been studied before any medication was given, after treatment with thiouracil had caused a fall in the basal metabolic rate to a standard level and after receiving both thiouracil and iodine. Observations have included microhistometric studies of biopsy specimens taken before either drug, after thiouracil treatment, but before iodine, and of glands removed at operation after treatment with both drugs.

Urinary excretion of radioactive iodine was determined before and during thiouracil treatment. Total and bound iodines were determined in the operatively removed thyroids.

The results show hyperplasia due to the disease before thiouracil,—average mean thyroid cell height 12.9 micra; and a greater degree of hyperplasia after thiouracil,—average mean acinar cell height 13.9 micra. However, involution was observed after iodine had been administered in addition to thiouracil, mean cell height 7.2 micra.

Before treatment with thiouracil the excretion of radioactive iodine averaged 25 per cent. Dur-

ing thiouracil treatment, the average excretion of radioactive iodine was 80 per cent.

The iodine values of thyroids removed at operation averaged for total iodine 22.1 mgm. per 100 grams of fresh thyroid tissue, and for thyroglobulin iodine 7.0 mgm. per 100 grams of fresh tissue. These values are comparable to the values obtained from the thyroids of thyrotoxic patients removed after treatment with thiouracil only.

CONCLUSIONS

It appears (1) that under thiouracil treatment the hyperplastic thyroid gland of Graves' disease becomes yet more hyperplastic, (2) that thiouracil prevents the utilization of iodine by the thyroid, (3) that notwithstanding this block to the collection of iodine produced by thiouracil, iodine causes involution of the thyroid gland in Graves' disease.

Therefore, it is concluded that iodine exerts 2 actions upon the thyroid gland in Graves' disease, an iodinating action and an involuting action, and that these 2 actions can be separated one from the other by means of thiouracil.

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CIRCULATION AND RESPIRATION DURING AN EPISODE OF CHILL AND FEVER IN MAN¹

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Changes in respiratory and circulatory function in patients with fever have been noted by clinicians for many centuries. In recent years a number of investigators have studied respiration and circulation in patients with febrile diseases (1), and many data bearing on these aspects of fever are available. Systematic studies on the reaction of the body to fever in different phases of a febrile reaction, however, have not been published. The present report is based on observations made upon patients experiencing an endogenous febrile reaction, *i.e.*, fever consequent to the introduction of foreign protein or microorganisms into the body. The effects of fever induced by physical means such as heating cabinets, hot baths, infrared radiation and diathermy are discussed elsewhere (1).

MATERIAL AND METHODS

Studies have been made on 8 subjects ranging in age between 25 and 63 years; 3 of them were women (Cases 1, 3, and 6). All were given fever for therapeutic purposes; the diagnoses included paresis (Case 2), rheumatoid arthritis (Cases 1, 3, 5, 6, 7), gonococcal arthritis (Case 4), and lymphedema of the face of unknown origin (Case 8). Fever was induced by means of the injection intravenously of a typhoid vaccine.

All studies were begun with the patients in the basal state; the patients remained in bed, taking no food and only small amounts of water throughout the period of observation on a given day. All measurements, except that of cardiac output, were made with the patient recumbent in bed; the cardiac output was measured with the patient propped up at an angle of about 30°. The pulse and respiratory rates were counted for 1 minute at frequent intervals. Arterial pressures were measured by the auscultatory method. The cardiac output was measured by the method of Starr and Gamble (2), the oxygen consumption, respiratory minute volume, alveolar air carbon dioxide content and respiratory quotient being determined at the same time. The arm-to-tongue circulation time was measured by means of sodium dehydrocholate (3), and the venous pressure was estimated by the

direct method of Moritz and von Tabora (4). Samples of blood taken from the femoral artery and from femoral and antecubital veins without stasis were analyzed for oxygen and carbon dioxide by means of the method of van Slyke and Neill (5) as modified by Fieser (6). The lung volume and its subdivisions were measured by the method of Christie (7), modified somewhat (8), the oxygen consumption being estimated at the same time. The method of Gibson and Evelyn (9) was used for measuring the plasma and blood volumes; the "direct" technique, using repeated injections of dye, and the "indirect" technique, using repeated observations after a single injection, were both employed. An attempt to study the function of lymphatics was made by means of a method suggested by Griffith (10), using the dye T-1824. The small blood vessels of the nailfold were studied by means of a microscope which magnified 100 times after cleansing the skin with xylol and application of liquid petrolatum to the area to be observed. The pH of the arterial blood was studied by means of a potentiometer with glass electrodes.

OBSERVATIONS

1. *Clinical findings.* A febrile episode was found to consist of 4 phases which have been designated: (1) *prodrome*, (2) *chill*, (3) *flush*, and (4) *defervescence*.

The *prodrome* began after the injection of the typhoid vaccine, lasted for 30 to 90 minutes and was characterized by a variety of complaints including lassitude, headache, aches and pains, and malaise. The *chill* phase developed rapidly and manifested itself by increasing pallor, greyish cyanosis, narrowing of all the superficial veins, hyperventilation, the appearance of a few beads of perspiration on the face and, at times, gooseflesh over the extremities also. Nausea occurred in almost all patients and was associated with vomiting in some. The rectal temperature rose, but the skin remained cool; the patients complained of feeling cold and exhibited a strong tendency to shiver. In some experiments this tendency manifested itself only by tensing of the muscles, while in others vigorous rigors occurred. If rigors developed, they could be abated by cov-

¹ This study was aided by a grant from the Josiah Macy, Jr. Foundation.

ering the patient, and if not present, they could be precipitated by exposing the patient to a blast of cool air or by placing a small piece of ice in his hand. Urine formation was minimal or absent during the *chill* phase. The *chill* phase lasted for

approximately an hour and one-half. It could be prevented by the previous administration of anti-pyretic drugs. The *flush* phase was characterized by the rapid development of a generalized flush which usually persisted but which might regress

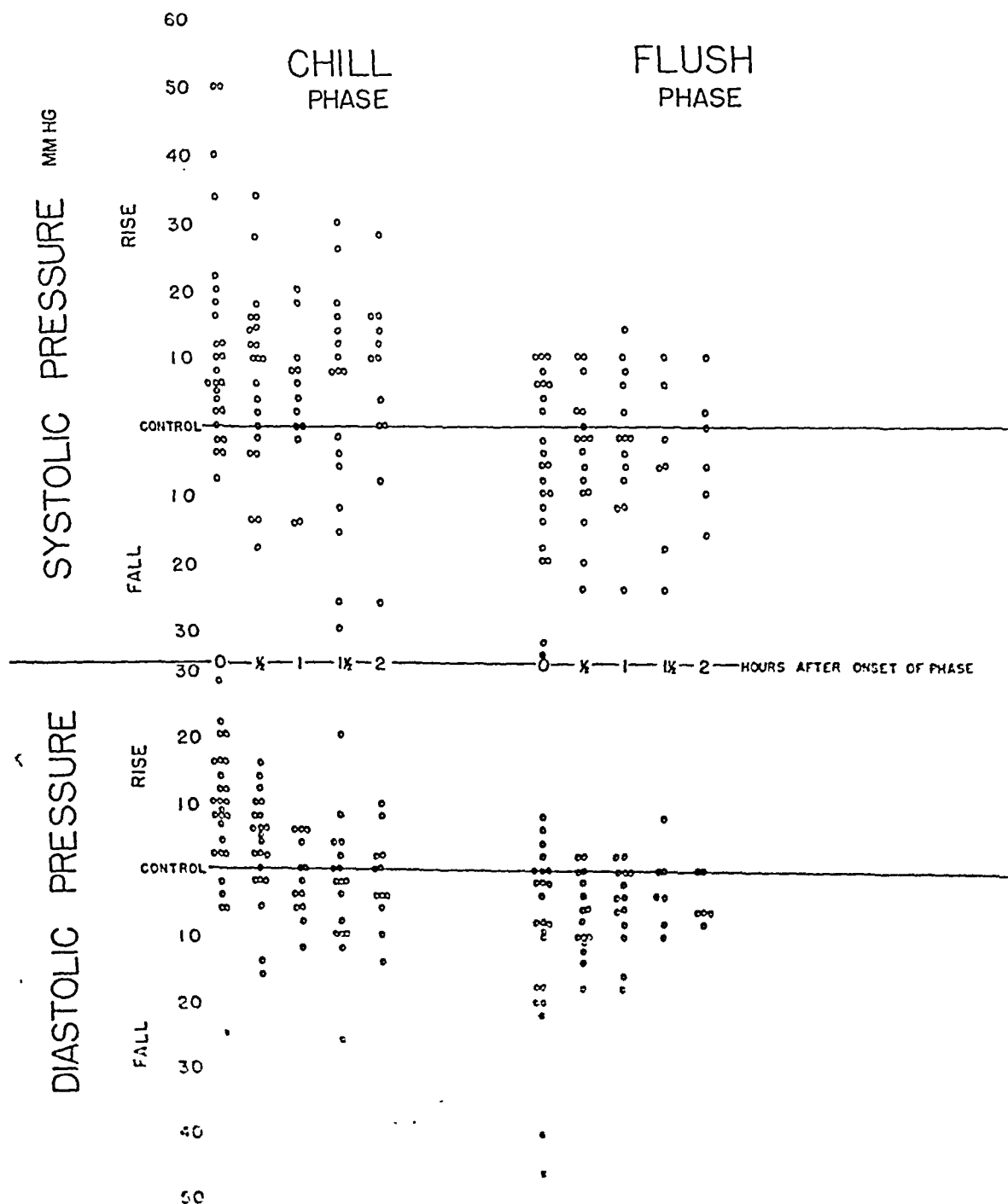


FIG. 1. CHANGES IN BLOOD PRESSURE IN ALL SUBJECTS DURING A FEVER REACTION

somewhat initially before becoming established. The cutaneous veins became greatly dilated. A drenching sweat often developed at or shortly after the appearance of the cutaneous flush, but in patients who had had a severe *chill* reaction the onset of severe sweating was often delayed for 10 to 20 minutes. At about the time of the establishment of the *flush* phase the patient complained of feeling warm and the skin felt hot; the rectal temperature remained at a high level with only minor fluctuations. A pounding headache was usually present. Nausea and vomiting were relieved with the onset of the *flush* phase. The onset of the *flush* phase was often associated with a diuresis. The *flush* phase lasted for approximately an hour and then passed gradually into the phase of *defervescence* when all abnormal findings regressed over a period of several hours. In a few instances, *defervescence* was interrupted by a second *chill* phase which instituted another typical febrile cycle.

2. *Cutaneous capillary microscopy.* No changes in cutaneous circulation occurred during the *prodrome*. The *chill* phase was ushered in by the rapid disappearance of most of the capillaries, the few remaining visible capillaries becoming greatly narrowed and containing blood moving abnormally slowly. The columns of blood in the capillaries appeared segmented. The vasoconstriction in some instances was at first variable, but soon became

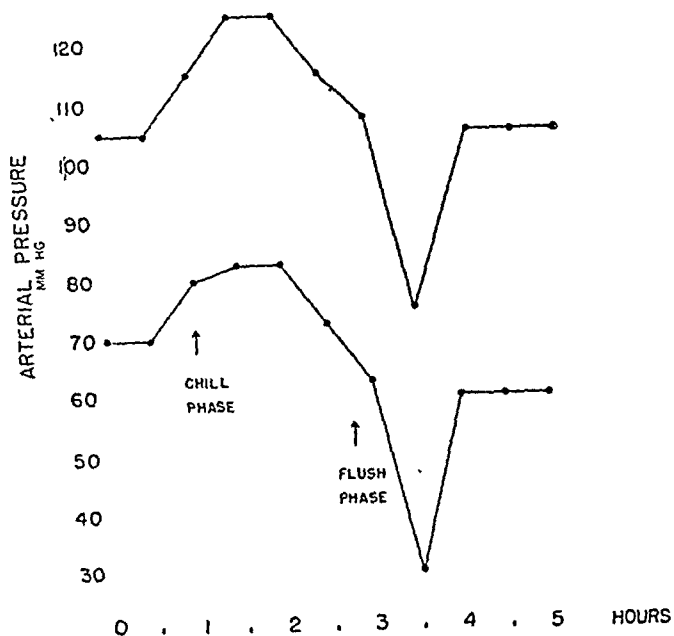


FIG. 2. INITIAL RISE IN ARTERIAL PRESSURE IN *Chill* PHASE AND MARKED FALL IN *Flush* PHASE

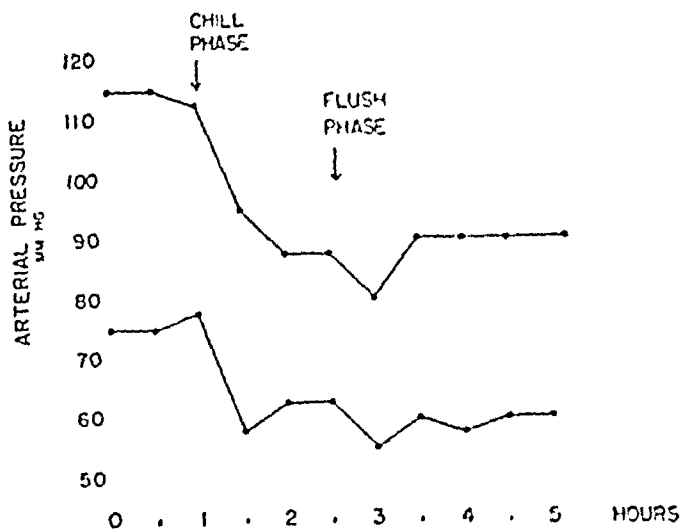


FIG. 3. FALL IN ARTERIAL PRESSURE IN *Chill* PHASE AND FURTHER DECREASE IN *Flush* PHASE

marked. In all instances it persisted until the onset of the *flush* phase. The latter was associated with the rapid appearance of large numbers of widely open capillaries containing blood moving very rapidly. All visible vessels, including arterioles and venules, appeared to pulsate. In some instances the onset of the *flush* phase was associated with the alternate appearance and regression of vasodilatation for a few minutes until finally the vasodilatation became established. *Defervescence* was characterized by a gradual return toward normal.

3. *Arterial pressure.* No changes occurred during the *prodrome*. A rise in systolic and diastolic blood pressure usually occurred in the early part of the *chill* phase, but this rise was not noted in 5 of 26 studies on the arterial pressure. In 7 experiments the arterial pressure fell below the control level late in the *chill* phase, the maximal decreases being 30 mm. Hg systolic and 26 mm. Hg diastolic. The onset of the *flush* phase was associated with a fall in arterial pressure, the maximal decrease below the control levels being 34 mm. Hg systolic and 46 mm. Hg diastolic. The arterial pressure gradually returned to normal during *defervescence* (Figures 1 to 3).

4. *Venous pressure.* No striking changes occurred, but a tendency toward lowering of the venous pressure became apparent during the *chill* phase (Table I), with resumption of normal levels during the *flush* phase.

5. *Cardiac output and arteriovenous oxygen difference.* The minute volume output of the

TABLE I
Venous pressure

Case	Venous pressure	Remarks	Rectal temperature
	cm. of H ₂ O		° F.
1	2.9	Control	99.2
	2.9	Early chill	99.6
	5.9	Moderate chill	101.3
	2.1	Moderate flush	102.0
2	9.3	Control	99.4
	3.3	Early chill	100.1
	3.1	Moderate chill	100.9
	3.5	Moderate flush	104.2
3	6.5	Control	99.3
	6.0	Early chill	101.7
	1.5	Severe chill	104.6
	2.5	Moderate flush	105.2
4	3.0	Control	99.3
	3.9	Moderate chill	103.3
	3.5	Moderate flush	105.7
	2.0	Defervescence	105.5
5	4.5	Control	99.0
	1.5	Severe chill	99.8
	3.5	Moderate chill	103.4
7	1.5	Control	99.1
	0.5	Moderate chill	101.5
	1.0	Moderate flush	103.5
8	4.7	Control	99.1
	2.0	Early chill	100.5
	1.8	Severe chill	101.2
	5.0	Moderate flush	103.5

heart and arteriovenous oxygen difference were normal before the onset of the *chill* phase in all 6 subjects studied (Table II). Changes in cardiac output were variable during the *chill* phase, increases occurring in half the cases. However, the increases in cardiac output which occurred were, except in 1 experiment, smaller than expected from the observed changes in oxygen consumption; in 2 of the 3 patients the cardiac output relative to oxygen consumption fell 14 and 15 per cent. In the patients in whom cardiac output did not rise at any time and fell only in the *chill* phase, the absolute decreases were between 12 and 33 per cent, and the decreases relative to oxygen consumption between 24 and 57 per cent. In the *chill* phase a variable increase in arteriovenous oxygen difference occurred, ranging from 1 to 131 per cent of the control value. In 5 of the 6 patients the arteriovenous difference increased by 16 per cent or more and in 1 instance a fall occurred. The amount of increase in arteriovenous difference varied roughly with the severity of the *chill* reaction. In the *flush* phase all pa-

tients exhibited an increase in cardiac output in excess of the rise in oxygen consumption; the absolute increase was between 45 and 182 per cent of the control value and the increase relative to oxygen consumption was between 10 and 86 per cent. The arteriovenous oxygen difference fell in all experiments by 9 to 46 per cent. The changes during the *flush* phase paralleled roughly the clinical manifestations of the reaction.

6. *Circulation time.* Slowing of the arm-to-tongue time occurred during the *chill* phase in 3 of the 4 patients studied (Table II). The slowing varied roughly with the severity of the *chill* reaction and ranged between 2 and 13 seconds, or 11 to 100 per cent, of the control level. In 1 of these the circulation time became accelerated somewhat late in the *chill* phase. The fourth subject showed an acceleration of 3 seconds, or 16 per cent, when studied in the *chill* phase. During the *flush* phase the circulation times became accelerated in all instances by 6 to 8.5 seconds, or 28 to 45 per cent of the control value. The changes during the *chill* and *flush* phases varied roughly with the severity of the reactions as judged by clinical criteria.

7. *Pulse rate.* A roughly linear relation existed between rectal temperature and pulse rate (Figure 4). The average rise was approximately 10 beats per degree of fever.

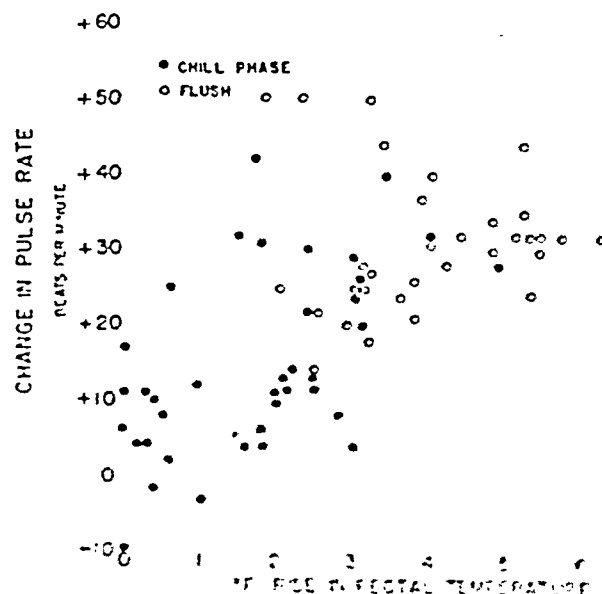


FIG. 4. CHANGE IN PULSE RATE DURING A FEVER EPISODE

TABLE II
Cardiac output and circulation time

Case	Cardiac output		Arteriovenous oxygen difference	Circulation time	Remarks	Rectal temperature
	<i>L. per minute</i>	<i>L. per 100 ml. of O₂ consumed</i>	<i>volumes per cent</i>	<i>seconds</i>		<i>° F.</i>
1	3.98	1.74	5.77	18	Control	98.9
	3.95	1.72	5.80	20	Early mild chill	99.6
	4.41	1.82	5.49	14	Late mild chill	101.9
	5.78	2.29	4.36	12	Moderate flush	102.8
	5.05	1.90	5.24	14	Defervescence	102.0
3	3.66	1.81	5.52	13	Control	99.3
	2.74	1.21	8.25	26	Moderate chill	102.5
	5.88	2.20	4.54		Moderate flush	101.0
4	4.46	1.66	6.02	19.5	Control	99.3
	3.92	1.27	7.84	25	Moderate chill	103.6
	2.89	0.72	13.93	31	Severe chill	105.6
	12.58	3.09	3.21	14	Severe flush	101.2
6	3.54	1.73	5.73		Control	98.0
	3.92	1.47	6.79		Mild chill	102.0
	6.90	2.34	4.26		Moderate flush	101.3
7	3.81	1.58	6.33		Control	98.7
	3.07	1.00	9.81		Severe chill	103.3
	6.78	2.10	4.75		Moderate flush	103.8
8	5.15	2.13	4.71	19	Control	98.7
	5.45	1.83	5.47	16	Mild chill	102.0
	8.01	2.34	4.28	10.5	Moderate flush	103.7

8. *Oxygen consumption.* In the absence of rigors or muscle tensing in the patients, the oxygen consumption varied roughly with the rectal temperature (Figure 5). The average rise in oxygen

consumption was approximately 7 per cent per degree of fever.

9. *Respiratory minute volume.* The respiratory minute volume rose during both the *chill* and *flush* phases (Table III). During the *chill* phase the hyperventilation exceeded the rise in oxygen consumption, while during the *flush* phase the respiratory volume relative to oxygen consumption per minute fell to or toward normal.

10. *Respiratory rate and tidal air.* The respiratory rate increased somewhat more than the minute volume during the *chill* phase, so that the tidal air fell slightly (Table III). During the *flush* phase the rate of respiration fell relative to the respiratory minute volume, and the tidal air volume rose to or somewhat above normal in all instances but 1 (Case 6).

11. *Respiratory quotient.* The respiratory quotient was between 0.78 and 0.83 before the body temperature rose. It was between 0.80 and 1.03 in the *chill* phase and between 0.81 and 0.86 in the *flush* phase.

12. *Alveolar air carbon dioxide content.* The alveolar air carbon dioxide content was between 5.0 and 5.6 per cent in the control and *prodromal*

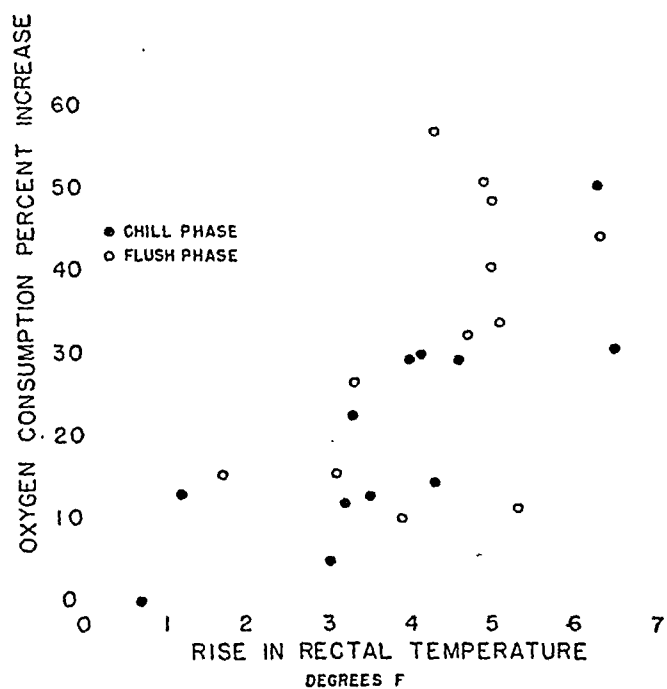


FIG. 5. INCREASE IN OXYGEN CONSUMPTION DURING A FEBRILE REACTION

periods (Table III). It fell to 3.9 to 5.2 per cent in the *chill* and was between 4.7 and 4.9 per cent in the *flush* phase.

13. *Subdivisions of the lung volume.* The functional residual (subtidal) air was increased by 2

to 24 per cent in the *chill* phase, the average increase being 12 per cent; in only 2 patients was the increase larger than the error of the method (Table IV). In the *flush* phase and *defervescence* the increases were between 1 and 16 per cent and

TABLE III
Respiratory dynamics

Case	Respiratory rate	Tidal air	Respiratory volume		Alveolar CO ₂	Remarks	Rectal temperature
	<i>per minute</i>	<i>ml.</i>	<i>L. per minute</i>	<i>L. per 100 ml. of O₂ consumed</i>	<i>per cent</i>		<i>° F.</i>
1	13	414	5.38	2.35	5.3	Control	98.9
	17	325	5.52	2.41	5.2	Early mild chill	99.6
	23	317	7.29	3.01	4.7	Mild chill	101.9
	18	400	7.20	2.86	4.6	Moderate flush	102.8
	15.5	427	6.62	2.50	4.6	Defervescence	102.0
3	16	328	5.25	2.60	5.5	Control	99.3
	21	309	6.50	2.88	5.0	Moderate chill	102.5
	19	349	6.63	2.47	4.9	Moderate flush	104.0
4	12	635	7.62	2.84	5.0	Control	99.3
	18.5	491	9.09	2.96	4.2	Moderate chill	103.6
	18	520	9.37	2.32	4.3	Severe chill	105.6
	16.5	683	11.27	2.79	4.3	Severe flush	104.2
6	10	457	4.57	2.23	5.6	Control	98.0
	25	261	6.51	2.45	4.6	Mild chill	102.0
	19	343	6.51	2.22	4.8	Moderate flush	104.3
7	13	525	6.81	2.83	5.5	Control	98.7
	23	518	12.41	3.97	3.9	Severe chill	103.3
	19	624	11.56	3.59	4.1	Moderate flush	103.8
8	12.5	516	6.45	2.65	5.1	Control	98.7
	17	505	9.09	3.05	4.2	Mild chill	102.0
	16	578	9.25	2.70	4.5	Moderate flush	103.7

TABLE IV
Subdivisions of the lung volume

Case	Functional residual air	Residual air	Reserve air	Complemental air	Vital capacity	Total capacity	Phase	Rectal temperature
	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>		<i>° F.</i>
1	1935	1320	615	2955	3570	4890	Control	98.9
	1965	1330	635	2800	3435	4765	Slight chill	100.0
	2005	1310	695	2935	3630	4940	Moderate flush	102.2
	1960	1285	675	2935	3610	4895	Defervescence	100.5
3	2330	1895	435	1390	1825	3720	Control	99.3
	2420	2125	295	1490	1500	3210	Severe chill	104.0
4	2535	1545	990	4365	5355	6900	Control	99.1
	2885	2070	815	3690	4505	6775	Severe chill	105.6
	2790	1820	970	4325	5295	7115	Severe flush	104.4
7	2610	2255	355	3250	3705	5560	Control	99.1
	2795	2555	240	3055	3295	5350	Severe chill	102.6
	2675	2300	375	3250	3625	5225	Moderate flush	103.4
8	1885	1230	655	3560	4215	5445	Control	99.3
	2340	1785	555	3010	3595	5160	Mild chill	101.6
	2180	1445	625	3410	4105	5060	Moderate flush	101.4

TABLE V
Blood oxygen and carbon dioxide content

Case	Arterial blood			Venous blood		Rectal temperature	Remarks
	Oxygen content	Oxygen saturation	Carbon dioxide content	Oxygen content	Carbon dioxide content		
1	<i>per cent</i> 15.23	<i>per cent</i> 100	<i>per cent</i> 44.68	<i>per cent</i> 11.48	<i>per cent</i> 47.39	° F. 99.7	Control
				9.92	46.45	100.5	Early chill
				5.93	39.09	102.0	Moderate chill
	15.43	94	40.52			102.4	Moderate chill
				14.79	41.78	103.4	Moderate flush
	15.29	99	41.11			104.5	Moderate flush
2				13.59	42.72		Defervescence
	18.32	94	43.43	12.72*	48.68	99.0	Control
	19.80	100	41.25	7.32*	49.23	100.1	Early chill
	19.07	98	40.01	9.97*	48.25	102.7	Moderate chill
				16.92	41.08	101.2	Moderate flush
3	19.96	94	41.30	14.64	42.55	99.6	Control
				4.89	46.90	101.1	Severe chill
	20.32	96	33.50			103.6	Severe chill
				15.70	36.32	104.7	Moderate flush
				16.93	39.00	103.7	Defervescence
4	18.63	90	42.95	10.23	49.41	99.2	Control
				4.10	49.60	102.5	Severe chill
	18.25	88	35.06	9.51	46.25	104.8	Early flush
				13.31	44.84	104.1	Severe flush
5	20.14	93	46.54	15.23	50.55	98.6	Control
	20.00	93	40.65	8.07	47.16	101.6	Severe chill
	19.20	89	40.88	17.65	44.43	105.0	Moderate flush
6	16.54	94	56.04	10.65	60.95	98.0	Control
				9.88	55.85	102.0	Mild chill
	15.06	86	50.87	12.14	54.87	102.6	Moderate flush
7				13.55	47.08	98.3	Control
				6.34	49.49	99.1	Early severe chill
				9.33	47.08	101.4	Severe chill
				8.94*	46.30	101.4	Severe chill
				9.25	44.30	103.7	Severe chill
				15.47	40.78	104.1	Moderate flush
				12.38*	44.15	102.1	Defervescence

* Femoral venous blood.

averaged 7 per cent. The volume of the residual air was somewhat increased during the febrile reaction in 4 of the 5 patients studied. During the *chill* phase the increases ranged between 1 and 45 per cent of the original values and averaged 23 per cent. It was outside the range of error of the method in all cases but one. During the *flush* phase the changes ranged between minus 3 and plus 21 per cent, averaging plus 10 per cent. The reserve (supplemental) air fell in 4 patients, the decreases ranging between 15 and 32 per cent in the *chill* phase and rose 13 per cent in 1 case. The average change was minus 13 per cent. In the *flush* phase no consistent change occurred. The complemental air was also diminished by 5 to 15

per cent, averaging 10 per cent, in the *chill* and showed no consistent change in the *flush* phase. The decreases in vital capacity were 0 to 18 per cent, averaging 11 per cent, in the *chill* phase; no consistent change occurred in the *flush* phase. The total lung volume showed no significant change at any time.

14. *Arterial blood gases.* Striking changes in arterial blood oxygen concentration did not occur in the patients studied; slight decreases in saturation were found in 2 who hyperventilated markedly (Table V). The arterial blood carbon dioxide concentration showed a considerable fall during the *chill* with little or no further change in the *flush* phase.

15. *Venous blood gases.* During the *chill* phase a fall in venous blood oxygen content, ranging between 0.77 and 9.71 volumes per cent, was observed; the amount of fall appeared to vary with the severity of the chill reaction (Table V). In the *flush* phase the venous blood oxygen content rose to levels 1.49 to 4.20 volumes per cent above the control values and approached the concentrations found in the arterial blood. The venous blood carbon dioxide content was variable during the *chill* phase, but fell in the *flush* phase by 3.16 to 7.60 volumes per cent below the control values. A return toward normal occurred during *defervescence*.

16. *Arterial pH.* Changes in the direction of alkalosis occurred in all patients studied (Table

TABLE VI
Arterial blood pH

Case	pH	Remarks	Rectal temperature
4	7.35	Control	° F. 99.3
	7.42	Chill	101.3
	7.44	Flush	104.7
	7.39	Defervescence	103.7
5	7.33	Control	98.6
	7.44	Chill	102.2
7	7.50	Control	99.6
	7.60	Early chill	100.7
	7.65	Late chill	103.0

TABLE VII
Measurements of hematocrit and plasma volume

Case	Hematocrit	Plasma volume	Remarks	Rectal temperature
1	percentage of cells	ml.		° F.
	45	2800	Control	100.0
	45	2820	Early chill	100.5
	45	2800	Moderate chill	102.8
4	45	2950	Moderate flush	103.5
	42	3475	Control	99.4
	42	3475	Prodrome	99.4
	43	3395	Early chill	102.4
	44	3175	Moderate chill	102.9
5	43	3210	Severe flush	105.5
	43	3560	Defervescence	103.4
5	38	4310	Control	98.6
	39.5	4300	Prodrome	98.6
	41	4170	Moderate chill	103.0
	39	4195	Moderate flush	103.0
8	41	2810	Control	99.1
	40.5	2910	Severe chill	102.0

TABLE VIII
Lymphatic function

Case	Area stained		Time	Streamers	Remarks	Rectal temperature
	Initial	Final				
7	mm.	mm.	minutes			° F.
	5×5	6×6	20	0	Control	99.4
	6×6	6×8	20	0	Prodrome	99.4
	5×5	7×7	20	0	Chill	100.8
	2×3	7×6	3	++++	Flush	102.4
8	5×5	6×6	20	0	Control	98.7
	5×4	5×6	20	0	Chill	102.0
	5×4	8×7	4	++++	Flush	103.5

VI). During the *chill* phase and in the absence of rigors, the pH rose 0.07 to 0.15. In the *flush* phase it was still elevated and fell during *defervescence*.

17. *Blood volume.* No changes in plasma volume or hematocrit occurred during the febrile episode (Table VII).

18. *Lymphatic function.* Wheals on the forearm containing the blue dye increased in size by 75 to 100 per cent slowly during a 20-minute period during the control period (Table VIII). Wheals made during the *chill* phase increased by the same amount or a little less. During the *flush* phase, however, a great increase in the size of the wheal occurred within 3 to 5 minutes; streamers radiating out from the wheal also became visible.

DISCUSSION

The clinical features of the various phases of a febrile reaction have long been recognized in a variety of infectious processes. Patients in whom a foreign protein has been injected pass through the entire course of the febrile reaction within a period of hours, so that comparative studies during the various phases may be made readily and with a minimum of variation consequent to uncontrolled extraneous factors. The endogenous febrile reaction studied under these circumstances gives rise to a series of clinical and physiological changes which occur in regular sequence and fall into uniform patterns.

The significance of the *prodromal* period is not clear, nor are the mechanisms which act during it clearly understood. This phase is characterized by such nonspecific complaints as headache, malaise, and lassitude; detectable changes in gas-

diovascular and respiratory dynamics do not occur. It is terminated by the onset of marked generalized vasoconstriction which is evident in the skin and assumed elsewhere because of the observed changes in arterial blood pressure. The narrowing or disappearance of cutaneous capillaries and the sluggish cutaneous flow observed here, and previously by others, was ascribed to marked arteriolar constriction (11, 12, 13). The conclusion that capillary blood pressure is unchanged during the *chill* phase of malaria (14) cannot be accepted because of the fact that the indirect method used is inaccurate. The elevation of arterial blood pressure which occurred in some of the experiments of the present study, as well as in those of other authors (14 to 17), indicates that arteriolar narrowing is widespread. More marked and persistent increases in arterial blood pressure would probably develop if it were not for the occurrence of changes in cardiac output which tend to lower arterial pressure. Not only are capillaries and arterial vessels constricted, but the visible veins also become markedly narrowed. It appears, therefore, that the vasoconstriction is a widespread phenomenon involving all vascular elements. On the other hand, some areas within the body probably do not participate in the fairly generalized vasoconstriction and indeed may be the seat of vasodilatation, for the circulating blood volume shows no change as measured here and as previously described (18). Studies on visceral circulation in an endogenous febrile reaction are scanty. A decrease in the size of the kidneys has been described (19) in animals in which a febrile reaction was induced, so that these organs are probably the site of vasoconstriction. Other authors have found a decrease in cerebral (20) and in renal (16) flow in the *chill* phase of induced febrile reactions. On the other hand, the findings of the present study which show an increase in the residual air volume and in the ratio of residual air volume to total pulmonary capacity in the *chill* phase suggest that some degree of pulmonary congestion may occur.

During the *chill* phase the cardiac output fell in some experiments and in the rest failed to rise in proportion to the increase in metabolic rate which occurred as the body temperature rose. This phenomenon was paralleled by increases in arterio-venous oxygen difference and by decreased oxygen

content of venous blood taken from the antecubital or femoral veins. None of the findings suggested direct depression of cardiac function as the cause of the observed absolute or relative decrease in minute volume output, i.e., bradycardia did not occur and the venous pressure did not rise. The tachycardia likewise did not reach levels which might cause lowering of the cardiac output. It is probable that the changes in cardiac output which occurred in the *chill* phase in the patients studied were the resultant of 2 opposing forces: (1) a tendency to increase parallel with the rising metabolic rate associated with fever; and (2) a tendency to decrease as a consequence of impairment of venous return due to intense vasoconstriction. In regard to the latter, it is of interest that the venous pressure tended to fall somewhat during the *chill* phase. One worker (14) had found no change in venous pressure during the *chill* phase of a malarial paroxysm. The arm-to-tongue circulation time was slowed early in the *chill* phase in every patient. Late in this phase, the circulation time in some patients became somewhat accelerated, while in others it remained slow. The initial slowing was probably consequent largely or entirely to slow flow secondary to cutaneous vasoconstriction, for cooling the skin has been shown to slow the arm-to-tongue time (21). During the early part of the *chill* phase changes in cardiac output are probably minimal or absent. Later in this period, however, the cardiac output may fall and when it does so, the circulation time becomes more prolonged. It is not possible to state how much of the prolongation in such instances is the effect of cutaneous vasoconstriction in the arm *per se* and how much is the consequence of diminished cardiac output.

Changes in respiration during the *chill* phase consist in increased respiratory minute volumes greatly in excess of increases in oxygen consumption due to the fever. There were also exaggerated increases in respiratory rate which resulted in slight or moderate decreases in tidal air volume. Several mechanisms may be responsible for the excessive hyperventilation of the *chill* phase. The above-described cutaneous vasoconstriction limits heat dispersal *via* the skin (22, 23), so that the effects of increased heat production are exaggerated and the body temperature rises rapidly. Increased temperatures within the brain give rise

to hyperventilation (24 to 27), presumably in an attempt to lower body temperature. Another factor which may increase respiratory minute volume out of proportion to metabolic requirements is the stasis which occurs when the cardiac output falls or at least fails to rise in proportion to oxygen consumption. Some degree of tissue anoxia is present in all such instances and in some experiments a rise in venous blood carbon dioxide also occurred. A third mechanism favoring excessive hyperventilation might be reflexes activated by pulmonary congestion. The importance of each of these causative factors cannot be evaluated. The respiratory changes which occurred in the *chill* phase resulted in a lowering of alveolar air carbon dioxide content and blowing off of appreciable amounts of carbon dioxide from the blood. The arterial blood carbon dioxide was significantly lowered, while the changes in the venous blood were the resultant of 2 opposing factors: (1) lowering of blood carbon dioxide consequent to hyperventilation; and (2) elevation of blood carbon dioxide consequent to stasis. Studies of respiratory quotient showed a tendency toward the giving off of larger amounts of carbon dioxide than in the basal state, but this was not definitely consequent solely to hyperventilation, since increased metabolism of carbohydrate also occurs. The loss of carbon dioxide from the blood gave rise to a tendency toward alkalosis; that alkalosis was not more marked is probably a consequence of the elevation of blood lactate and pyruvate levels which occurs in fever (28).

The marked generalized vasodilatation which characterized the *flush* phase presumably involved all vascular elements. Microscopy revealed the capillaries of the skin to be markedly increased in number and size and direct inspection showed the superficial veins to be dilated. Arteriolar dilatation probably also was present, for some fall in arterial blood pressure and the appearance of capillary dilatation occurred regularly. In addition, arteriovenous shunts probably became patent, for venous blood became arterialized, often markedly so. The slight rise in venous pressure which was noted might also have been consequent to opening of arteriovenous channels. Nevertheless, vascular constriction must have occurred somewhere in the body, for the circulating blood volume was unchanged. Increases in cardiac output

in the *flush* phase were out of proportion to the metabolic needs, and the arm-to-tongue time was greatly accelerated. The renal flow may also increase in the *flush* phase (16, 19, 30). These phenomena may have been consequent, at least in part, to increased venous return secondary to opening of the arteriovenous shunts. Another factor making for this increase in cardiac output is elevated cerebral temperature (25). The absence of stasis in the *flush* phase and the restoration of 1 of the mechanisms for heat dispersal lost in the *chill* phase, *i.e.*, increased circulation through the skin, removes 2 factors which exaggerate the hyperventilation of the *chill* phase. Diminished pulmonary congestion has the same effect. Accordingly, the respiratory minute volume is lowered relative to oxygen consumption and returns to or toward a normal relation with metabolic rate. Loss of carbon dioxide from the arterial blood and alkalosis, therefore, do not progress.

A state which had the clinical characteristics of shock developed in patients in whom the severest *chill* reactions occurred. The patients were pale, cold, sweaty, anxious, and dyspneic; they showed tachycardia and a falling arterial blood pressure. Physiological studies made at this time revealed very low cardiac output, marked deoxygenation of venous blood, and a fall in venous pressure. This state differed from that seen in other types of shock in that blood volume, oxygen consumption, and rectal temperature did not fall; the syndrome was, in addition, self-limited. The clinical manifestations of shock were in some experiments temporarily exaggerated at the onset of the *flush* phase when vasodilatation rapidly developed.

The observations on lymphatic function made here are difficult to interpret. It appears that lymphatic flow is increased in the *flush* phase of the febrile reaction, but the mechanisms responsible for this change are difficult to evaluate. Vasodilatation and elevated capillary pressure tend to increase filtration from the blood. The former obviously occurs and the latter may also. A rise in capillary pressure when the skin is heated locally has been demonstrated (31). Whether such a rise can also occur in a febrile reaction when vasodilatation is generalized and may be extreme cannot be determined.

The factors which initiate the febrile reaction and determine its phases are not clearly understood. The cause of the increased heat production which occurs in a febrile reaction is probably related in some way to hypothalamic function, for damage to that area prevents it (32). Similarly, the vasomotor manifestations of the febrile reaction appear to be initiated by the hypothalamus. The onset of flushing and sweating occurs at a higher level in patients undergoing a febrile reaction than in subjects exposed to warm environmental temperatures and, accordingly, it appears that thermostatic control of the peripheral vascular and sweating mechanisms is reset at a higher than normal level in endogenous fever.

SUMMARY AND CONCLUSIONS

1. Changes in circulation and respiration have been studied in 8 patients following the injection intravenously of typhoid vaccine.

2. The febrile reaction consisted of 4 phases: (a) *prodrome*, (b) *chill*, (c) *flush*, (d) *defervescence*. Each had distinctive clinical features and, except for the prodrome, each was characterized by a definite series of physiological phenomena.

3. The *chill* phase was characterized by intense vasoconstriction, decreased cardiac output, slowed circulation time, hyperventilation, alkalosis, and oliguria. There was an initial hypertension followed by hypotension and, in some experiments, the development of shock.

4. The *flush* phase was characterized by marked vasodilatation, drenching sweats, increased cardiac output, accelerated circulation time, a lesser degree of hyperventilation, maintained alkalosis, diuresis, and in many experiments a sudden fall in blood pressure at the onset of the phase.

5. *Defervescence* was characterized by a return of all mechanisms toward normal.

6. Lymphatic function was apparently increased in the *flush* phase.

7. No change in circulating plasma volume occurred during any phase of the febrile reaction.

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THE ESTIMATION OF HEPATIC BLOOD FLOW IN MAN

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A method of measuring hepatic blood flow is required in the quantitative analysis of hepatic function. Without this measurement, changes in hepatic function due to hemodynamic factors cannot be differentiated from those due to altered cellular activity, and metabolic exchanges cannot be accurately evaluated. Although various direct methods (1 to 7) have been devised and used in animals, the surgical manipulation necessary renders all such methods inapplicable to man. This paper describes a method based on the "Fick principle" by which hepatic blood flow has been estimated in man without trauma or anesthesia.

The "Fick principle" may be applied to any organ provided 3 facts are known: (1) the concentration of some substance, X, in the blood entering the organ, (2) the concentration of X in the mixed venous blood leaving the organ, and (3) the total amount of X removed from the blood by the organ each minute. Given these data, the blood flow through the organ per minute may be calculated by dividing the total removal rate of X by the amount of X removed from each milliliter of blood as it traverses the organ. In theory, this principle may be applied to the measurement of the blood flow through the human liver.

In practice, the concentration of X in the blood entering the liver cannot be determined unless X is a substance which may be assumed to exist in equal concentration in the afferent hepatic blood and in the peripheral venous or arterial blood. The concentration of X in the blood draining from the liver can be determined in hepatic venous blood obtained by an application of the venous catheterization technique (8). Since rapid and quantitative collection of bile is not feasible, the removal rate of X by the liver cannot be measured directly. An indirect method, however, is available if X can be given at such a rate that its blood level remains constant. Under these conditions, the infusion rate equals the hepatic removal rate, provided the extraction of X depends en-

tirely upon hepatic activity. Bromsulphalein (BSP) was used as the test substance, X, because it appeared to satisfy the above conditions.

EXPERIMENTAL PROCEDURE

After the subject¹ assumed the supine position on the fluoroscopic table, a catheter,² through which an isotonic saline infusion flowed, was inserted into the median basilic vein (8). Under fluoroscopic control, the catheter was passed into the superior vena cava and thence into the right atrium. The valve of the inferior vena cava could usually be avoided by directing the catheter tip to the right and posteriorly during its passage through the atrio-caval orifice. Once beyond this valve the tip was brought to bear more laterally and directed into the veins draining the right hepatic lobe. Catheterization of the left hepatic veins was somewhat more difficult. For the purposes of the present study, a right hepatic vein was catheterized on every occasion and the tip placed approximately in the center of the lobe. The catheter was kept in place for as long as 3 hours without untoward symptoms.

An intravenous infusion of isotonic saline containing BSP was next started in one of the lateral veins of the arm used for catheterization. A sample of the infusion was always taken for analysis. The rate of inflow, under the force of gravity, was controlled by a tunnel clamp (made by the Harvard Instrument Company) at 4 ml. per minute, measured with a calibrated Murphy drip (15 drops per ml.). By this device the inflow rate could be maintained remarkably constant, although it varied occasionally as much as 0.06 ml. per minute. The BSP concentration of the infusion was usually adjusted so that approximately 3.0 mgm. were delivered per minute per

¹ The 23 subjects examined in this study were drawn for the most part from a youthful group under penicillin therapy for primary or secondary syphilis. Except for syphilis, they appeared to be in good health and, as far as could be judged clinically, had no evidence of hepatic disease. All had had sufficient therapy to induce healing of lesions involving the skin or mucous membranes. The subjects were examined in the resting state and were either fasting or at least 3 hours post-prandial.

² The catheters used in this study were designed and manufactured for use in this and other studies by the United States Catheter and Instrument Corporation, Glenn Falls, New York. They are 100 cm. in length and terminate in a slightly-curved whistle tip ("Cournand Tip") with 1 "eye."

TABLE I

Estimated hepatic blood flow in man

(All data in this table were obtained under standard conditions in resting subjects at least 3 hours post-prandial. Only studies in which the serum level of bromsulphalein was constant or changing no more than 0.0005 mgm. per ml. per minute are presented.)

Subject	Sex	Age	Surface area	Peripheral serum concentration BSP	Extraction BSP	Total removal rate BSP	Hepatic blood flow	Hepatic blood flow
		years	M. ²	mgm. per cent	per cent	mgm. per minute	ml. per minute	ml. per minute per 1.73 M. ²
1	C. M.	M	57	1.50	1.13	83.4	7.30	1350
2	D. C.	M	30	1.50	0.75	75.5	4.90	1340
3	J. B.	M	22	1.98	0.51	90.1	5.28	2110
4	S. G.	M	29	1.93	1.21	34.4	5.00	1905
5	M. B.	M	21	1.68	0.77	72.0	5.00	1555
6	V. C.	M	23	1.94	0.80	70.8	6.00	2055
7	J. F.	M	23	1.77	1.67	43.7	5.72	1185
8	C. H.	M	29	1.74	1.08	47.0	4.80	1640
9	P. W.	F	36	1.49	0.90	57.5	3.88	1190
10	S. J.	M	28	1.60	1.21	48.3	4.27	1175
11	W. T.	M	39	1.85	0.85	64.0	4.80	1650
12	R. P.	M	20	1.90	0.87	70.0	5.12	1535
13	L. K.	M	30	2.06	0.94	73.4	5.72	1920
14	D. D.	M	56	1.60	0.90	43.4	3.36	1455
15	M. T.	M	36	1.55	0.50	88.0	4.20	1500
16	L. C.	M	22	1.78	0.65	84.0	5.25	1635
17	A. A.	M	36	1.91	1.29	73.0	5.86	1200
18	J. M.	M	25	1.99	*	*	*	1460
19	A. D.	M	20	1.85	1.21	64.8	5.98	1360
20	L. W.	F	20	1.62	0.97	60.2	5.44	1485
21	M. S.	F	40	1.61	0.69	84.4	4.17	1296
22	N. O.	F	35	1.60	*	*	*	1523
23	V. B.	F	19	1.53	*	*	*	1191
Average								1497

* In these studies hepatic blood was obtained at various sampling sites. The figures for EHBF are averages of 3 or more values. See Table II for details.

square meter of body surface. Faster rates of delivery often resulted in a rising BSP serum level. A priming dose of BSP (150 mgm.) was given to 15 subjects (J. F. to M. S., Table I) to raise the serum level quickly to about 1 mgm. per cent and to shorten the time of equilibration.

Samples of blood were obtained, as a rule, at 10-minute intervals over a period of an hour or more. After the salt solution in the venous catheter was washed out by withdrawing several ml. of blood, 8 to 15 additional ml. were collected in a clean, dry, sterile syringe. Simultaneously, a sample of blood was withdrawn from the vein or artery of the opposite arm.

The BSP concentration in the serum was determined colorimetrically. One or 2 ml. of serum was pipetted into an Evelyn colorimeter tube and the volume made up to 10 ml. with isotonic sodium chloride solution. If the concentration of BSP in the serum was estimated at less than 1.5 mgm. per 100 ml., 2 ml. of serum was used; if more than 1.5 mgm. per cent, 1 ml. sufficed. With concentrations over 4 mgm. per 100 ml., 1 ml. serum was made up to 20 ml. in order to obtain good readings. The diluted serum was analyzed in the macro-unit of an Evelyn photoelectric colorimeter with a 580 filter. After a "central setting" was obtained, 2 drops of concentrated

NaOH were added, the mixture well agitated, and a reading taken within 1 minute of adding the alkali. The BSP concentration was obtained from the galvanometer reading by using a calibration curve, and by correction for the dilution. All determinations were made in duplicate.*

* A complete evaluation of the method is to appear elsewhere, but the following points are of importance:

(1) For calibration, weighed samples of pure bromsulphophthalein powder (H. W. and D.), dissolved in 0.85 per cent sodium chloride solution, were diluted to concentrations ranging between 0.05 and 0.8 mgm. per 100 ml. Galvanometer readings were made as described above, and the results plotted on semi-logarithmic paper against the known concentrations. A straight line relationship was obtained up to a concentration of 0.4 mgm. per 100 ml.; beyond this point, the line showed a marked tendency to flatten out. For this reason, all the samples were diluted to bring the concentration of BSP below 0.4 mgm. per 100 ml.

(2) The addition of concentrated NaOH to diluted serum not containing BSP reduced the "central setting." This decrease was minimal if the serum was clear and pale, but was pronounced if protein hemolysis or turbidity were present. Consequently, samples which were greatly

Calculation of hepatic blood flow

Figure 1 sets forth diagrammatically the procedure followed in estimating hepatic blood flow. Peripheral and hepatic serum concentrations of BSP were plotted on semi-logarithmic paper against time. Straight lines were drawn between these points (P—peripheral venous concentration of BSP and H—hepatic venous concentration, Figure 1). Values of P and H used in the calculation of hepatic blood flow were then obtained by interpolation from the curves at a time midway between measured values of P (cross-marks connected by interrupted line on P and H curves, Figure 1). With constant peripheral plasma levels (Figure 1), the total BSP removal rate, R, was taken as equal to the rate of infusion in mgm. per minute. Then taking EHBf as the estimated hepatic blood flow, P as the concentration of BSP in mgm. per 100 ml. in the afferent hepatic blood, and H as the concentration in the efferent hepatic blood:

$$\text{EHBf} = \frac{R}{0.01(P - H)} \times \frac{1}{1 - \text{hematocrit}}$$

Hepatic blood flow may also be estimated if the levels of BSP in the peripheral blood are changing, but this

hemolyzed or turbid were discarded. In other samples, the effect of alkali on the serum presumably introduced a small error which lowered the BSP readings below their actual value.

Known quantities of BSP, when added to pale, clear sera, were recovered with less than a 5 per cent error. In more turbid or pigmented sera, recovery of strong BSP solutions was similarly satisfactory; but from weaker solutions (*i.e.*, 0.2 to 1.0 mgm. BSP per 100 ml.) recovery was only 85 to 95 per cent. In the range of BSP concentration used in the study of hepatic blood flow, the absolute error was never more than 0.1 mgm. per 100 ml. Since this error probably affected hepatic and peripheral venous blood similarly, the calculations of estimated hepatic blood flow were not significantly influenced.

(3) Samples of serum containing BSP in its colorless state could be kept in the ice box for 4 days without apparent loss of dye. Once alkali had been added, however, whether to diluted or undiluted serum, the color thus developed diminished in intensity very slowly.

(4) BSP was not contained in washed red cells. Serum proved easier to use than plasma. Although BSP in saline solutions dialyzed freely across a cellophane membrane, no dialysis occurred if BSP was dissolved in serum. BSP in serum thus appeared to be completely bound by protein. According to salting-out experiments, this linkage seemed to be preponderantly with the albumin fraction. No method of precipitating proteins (by acetone, salting-out, etc.) that does not decrease the quantity of recoverable BSP has been found.

(5) If the concentration of BSP in the undiluted serum was over 0.5 mgm. per 100 ml., duplicate readings checked within 5 per cent. With serum concentrations over 1.0 mgm. per 100 ml., duplicate readings checked within 3 per cent and usually yielded identical results.

estimate requires the measurement of plasma volume. When serum levels are rising, it is obvious that less dye is removed than is administered. Hence, the removal rate is equal to the rate of administration less the rate of retention. Taking I as the rate of infusion of BSP in mgm. per minute, R as the total removal rate of BSP in mgm. per minute, V as the total plasma volume in ml., and ΔP as the rate of change in the concentration of the dye in the peripheral serum in mgm. per ml. per minute,

$$R = I - (\Delta P \times V)$$

when the blood level is rising (Figure 1). When the peripheral level is falling (Figure 1):

$$R = I + (\Delta P \times PV)$$

The change in the peripheral serum level (mgm. per ml.) during the minute immediately preceding the midpoint was taken as ΔP . Thus the difference between the concentrations per ml. at the two cross-marks on curve P (Figure 1) is equal to ΔP . Plasma volume (V) was estimated from the tables of two other investigators (9). This procedure may introduce a small error that depends upon the deviation of the subject from the average.

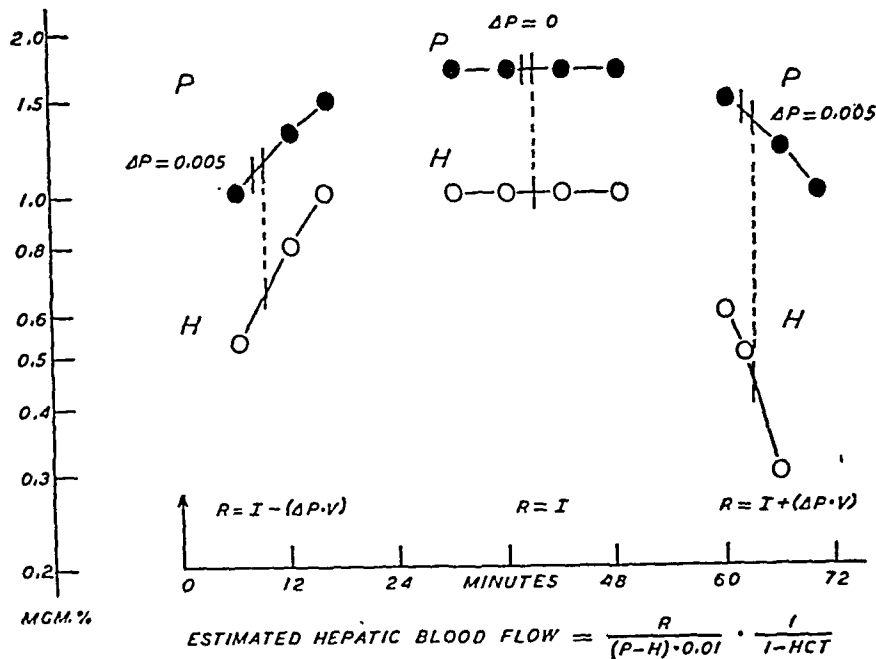
In this report, hepatic blood flow was calculated only from data obtained when the level of BSP in the peripheral blood was constant or changing less than 0.0005 mgm. per ml. per minute. Two studies have been excluded from the results because hemolysis and very low plasma levels of BSP caused analytical difficulties.

Validity of the method

The validity of the method hinges upon 4 basic assumptions; *viz.*, (1) bromsulphalein is removed from the blood exclusively by the liver, (2) the concentration of the dye in the peripheral venous blood corresponds to the concentration in the blood entering the liver, (3) the BSP level in a sample of blood taken from a right hepatic vein is representative of the level in total pooled hepatic venous output, and (4) the basal hepatic blood flow is not altered by BSP in the blood nor by the catheter in the liver.

Although it seems generally agreed that bromsulphalein is removed from the blood chiefly by the liver, the location of intrahepatic and extrahepatic uptake sites remains disputed. Certain investigators favor the view that BSP is taken up by the reticulo-endothelial system both within and without the liver because removal of the dye is retarded when cells of this system are blocked by India Ink or removed by splenectomy (10, 11). The method of determining hepatic removal rate used in this study is not materially affected, however, whether BSP is removed entirely by the

CALCULATION OF ESTIMATED HEPATIC BLOOD FLOW



R — BSP REMOVAL RATE MGM./MIN.

P — CONCENTRATION BSP IN PERIPHERAL SERUM MGM./100 CC

H — CONCENTRATION BSP IN HEPATIC SERUM MGM./100 CC

I — INFUSION BSP MGM./MIN.

V — PLASMA VOLUME CC

ΔP — CHANGE IN PERIPHERAL CONCENTRATION BSP MGM./CC/MIN.

FIG. 1

Serum concentrations (mgm. per 100 ml.) of BSP in peripheral venous blood (*P*) and in hepatic venous blood (*H*) are plotted on semi-logarithmic paper against time. Concentrations used in the calculation of EHBF are obtained by interpolation at the midpoint between measured concentrations on *P* (right-hand cross-mark of each pair on *P*), and at the same time on *H* (cross-mark on *H*). The difference between these 2 values (*P*−*H*) multiplied by 0.01 yields the amount of BSP removed from each ml. of serum as it passes through the liver. Each ΔP was taken as the difference between BSP concentrations per ml. of serum at the midpoint and 1 minute prior to the midpoint on *P* (cross-marks on *P*).

liver or partially by reticulo-endothelial cells elsewhere in the body.

The extrahepatic portions of the reticulo-endothelial system by which BSP could be withdrawn from the blood lie principally in the portal circulation and in the bone marrow. Since the blood draining from the portal vein, except for a small volume lost through collateral channels, is emptied into the inferior vena cava by way of the hepatic veins, the portal circuit is, in effect, an extension of the hepatic vascular bed. Hence, removal of the dye by the spleen or gastrointestinal tract is

good reason to believe that this possibility is unlikely. In the first place, the volume of blood flowing to the bone marrow probably is small and the amount of BSP available for removal by this route, therefore, should be minimal. Furthermore, as long as the serum concentration of BSP remains unchanged, the reticulo-endothelial cells of the bone marrow presumably become saturated with respect to that concentration and remove no further dye.

Removal by the kidney is very small in normal subjects, amounting to no more than 12 mgm. per hour when the blood level is maintained at 1 mgm. per 100 ml., but losses by this route may be large if proteinuria is present (12). The dye is completely bound to the plasma proteins and does not dialyze from plasma through cellophane membranes. It is unlikely, therefore, that significant amounts of the dye are lost from the blood stream by diffusion into extravascular water. Moreover, it has not been found in ascitic fluid even when present in the blood in high concentration (12). A small amount of BSP may be destroyed in the body, but this activity is probably negligible.

Finally, no evidence of significant removal of the dye elsewhere in the body has been obtained in these studies. Concentration of BSP in blood

(d) the rate of infusion is 6 mgm. per minute; and (e) the hematocrit is 50 per cent. Then:

$$\text{EHBF} = \frac{6}{0.01 \times 0.8} \times \frac{1}{0.5} = 1,500 \text{ ml. per minute}$$

(2) Assume, on the other hand, that splenic activity accounts for 1.2 mgm. per minute of the total removal rate, and that 300 ml. of blood (150 ml. serum) passes through the spleen per minute. If the splenic venous return drained directly into the peripheral venous system, the calculations would yield a falsely high hepatic blood flow, for the total removal rate would remain at 6 mgm. per minute, while the peripheral-hepatic venous concentration difference, deprived of its splenic component, would be reduced. The spleen, however, drains through the liver. Hence under the conditions assumed above, 1,500 ml. of blood reaching the liver per minute through the portal vein would contain 600 ml. of serum with a BSP concentration of 1.4 mgm. per 100 ml. and 150 ml. serum coming from the spleen containing 0.6 mgm. BSP per 100 ml. The P-H difference due solely to hepatic activity would then equal 0.64 mgm. per cent and:

$$\text{EHBF} = \frac{4.8}{0.01 \times 0.64} \times \frac{1}{0.5} = 1,500 \text{ ml. per minute}$$

the same figure derived under assumption (1).

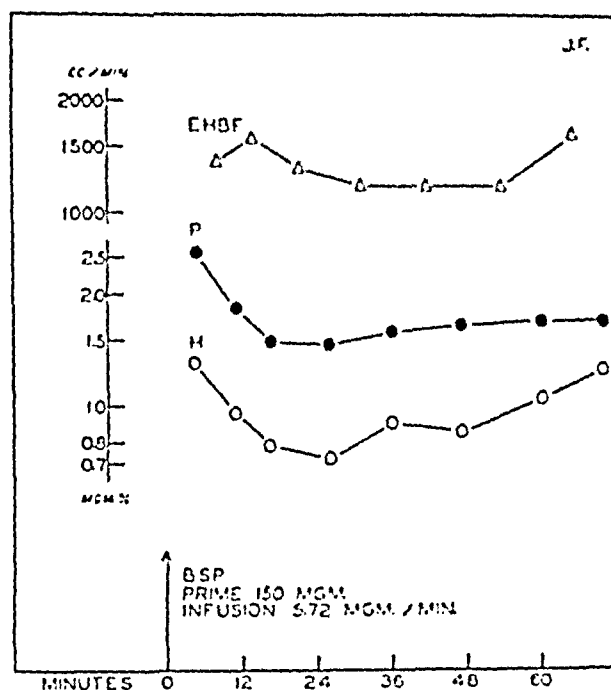


FIG. 2. ILLUSTRATIVE STUDY OF ESTIMATED HEPATIC BLOOD FLOW IN MAN

Subject J. F., a 23-year-old, white male, had completed a course of penicillin therapy for primary syphilis. Concentrations of bromsulphalein in the peripheral venous serum (P) following intravenous administration of the dye at the arrow are plotted as closed circles. The concentrations of BSP in hepatic venous serum (H) are plotted as open circles. The estimated hepatic blood flows (EHBF) are plotted as open triangles. EHBF does not change greatly over a period of more than 1 hour.

taken simultaneously from an antecubital vein, the brachial artery and the inferior vena cava has been found to differ by no more than 5 per cent.

In summary, then, it appears that the amount of bromsulphalein lost by destruction, by diffusion, by extraction in the bone marrow, and by way of the urine is not large enough to invalidate the first assumption; *i.e.*, the removal of BSP can be considered equal to the infusion rate when the level of dye in the peripheral blood remains constant.

Since the concentration of BSP in peripheral arterial and venous blood exhibits no significant difference, blood taken from an antecubital vein should contain the same proportion of dye as arterial blood entering the portal system and the liver. Although portal organs other than the liver may remove BSP, it has been shown that the inclusion of portal blood in hepatic venous outflow cancels any error introduced in this manner. For

these reasons, the concentration of BSP in a peripheral vein has been considered equivalent to the dye content of blood entering the liver.

Mixed hepatic venous blood cannot be obtained in man because the hepatic veins empty separately into the inferior vena cava. However, blood draining from a large portion of the liver may be sampled. In this study, blood has been taken from one of the right hepatic veins. Obviously, data obtained from such localized sampling is not satisfactory for calculating the true blood flow of the whole liver. The results presented in Table I, therefore, have been designated "estimated hepatic blood flow" (EHBf).

To what extent EHBf may deviate from true blood flow cannot be ascertained without a means of sampling total pooled hepatic venous blood. The magnitude of deviation may be judged, however, by comparing BSP extraction at different

locations in the same liver.⁵ Table II sets forth the data obtained in 3 such studies. The observed variation in BSP extraction may be explained in part by reflux of blood from the inferior vena cava since extraction appeared to decrease in several instances as the caval orifice was approached. This source of error may be avoided by placing the catheter tip several centimeters beyond the orifice and by withdrawing blood only when the subject is breathing quietly. A more fundamental cause of the unequal BSP extractions may be the fact that different areas in the liver contain varying proportions of capsular, portal, and other non-extracting tissue.

⁵ Extraction of BSP by the liver may be expressed as the percentage of dye removed from each ml. of blood as it passes through the liver; i.e., the difference between BSP serum concentrations in the peripheral and hepatic venous blood divided by the serum concentration in the peripheral blood.

TABLE II

Bromsulphalein extraction and EHBf at various hepatic sampling sites. The symbols mark the position of the catheter tip in the liver during sampling

Subject	Position of catheter in liver	BSP peripheral serum	Extraction BSP	BSP total removal rate	Estimated hepatic blood flow		Range
		mgm. per 100 ml.	per cent	mgm. per minute	ml. per minute	ml. per minute per 1.73 M. ²	ml. per minute per 1.73 M. ²
J. M.		▽ 1.20	86.2	6.17	1088	945	
		○ 1.03	74.6	6.00	1423	1238	
		● 0.91	66.2	6.17	1860	1618	673
N. O.		▽ 1.24	49.4	4.64	1286	1390	
		○ 1.23	39.4	4.82	1696	1832	
		▽ 1.08	49.1	4.75	1530	1655	442
		● 1.07	47.4	4.75	1520	1718	
V. B.		▽ 1.50	43.0	4.85	1365	1546	
		○ 1.45	46.0	4.92	1315	1436	
		▽ 1.41	55.6	4.92	1140	1290	
		● 1.37	69.1	4.92	941	1061	451

For any individual liver, the figure for true blood flow probably falls somewhere within the range of EHBF calculated at different sampling sites, and the range thus permits an estimate of the experimental error. In 3 subjects, this range was 442, 481, and 673 ml. per minute respectively (Table II). In other studies in which the position of the catheter was less rigidly controlled, the range of blood flow in any 1 subject never exceeded 800 ml. per minute. Hence, any observed EHBF may be said to approach true blood flow within the limits of this range. It is of interest that the range of EHBF as recorded in a group of individuals (Table I) also falls within 800 ml. per minute.

Certain dyes may affect hepatic blood flow (13). The effect of BSP in this respect has not been determined, but no relationship was noted in this study between EHBF and the serum concentration, total dose, or the rate of infusion of BSP. However, the possibility of an immediate maximal response of the hepatic vasculature to minimal amounts of BSP cannot be eliminated.

Passage of the catheter through the peripheral veins often induced a venous spasm which usually was fleeting, but at times was strong enough to impede catheterization. A similar reaction apparently occurred in the liver in some instances when the catheter was placed in the smaller radicles of the hepatic vein. On these occasions, the subject complained of epigastric pain which was immediately relieved by withdrawal of the catheter. A definite sense of resistance, possibly due to spasm of the vein, was noted by the operator during the withdrawal. As a rule, however, the catheter was passed without difficulty into many hepatic venous radicles and deep into the substance of the liver without evidence of spasm. Since hepatic venospasm occurred infrequently and was always transient, it is unlikely that the figures presented in Table I were affected in any way by such a reaction.

COMMENT

Study of the circulation of blood through the lobular sinusoids by means of the quartz rod illumination technique revealed an intermittent and irregular rhythmicity of the intrahepatic blood flow of anesthetized animals (14). If such an irregular intermittency of hepatic blood flow were

characteristic of relatively large sections of the human liver, BSP extraction might be expected to fluctuate widely from moment to moment. It was demonstrated repeatedly, however, that the percentage of BSP extracted from the blood obtained at a single sampling site in the liver remained constant for periods of 1 hour or more. If intermittency occurs in man, it must occur in very small vascular units alternating in a purely random manner.

The constancy of BSP extraction also indicates the absence of shunting activity in the human liver under the conditions of this study. The low extraction percentage (less than 60 per cent) found in 7 subjects may have been caused, it is true, by sustained shunts. However, no direct anastomoses between the portal and hepatic venous circuits have been demonstrated anatomically in man (15), and it is possible that the low extraction percentages in these instances arose from saturation of hepatic BSP removal mechanisms.

Since it appeared that 75 per cent of the intrahepatic circulation in animals was inactive at any moment, a "reserve" permitting 4-fold expansion of hepatic circulation and function has been postulated (14). In accord with this hypothesis, a direct method (7) of measuring hepatic blood flow in the dog has yielded a range of 40 to 160 ml. per 100 grams of liver tissue, with an average of 100 ml. per 100 grams. The average figure for EHBF in ideal man (surface area—1.73 M.²) was 1,497 ml. per minute or (assuming average normal liver weight) 100 ml. per 100 grams of tissue, a figure in complete agreement with that found in the dog. This finding gives weight to the view that average EHBF approximates the average true hepatic flow. The limited area of variation (1,085 to 1,845 ml.), however, stands in marked contrast to data derived from animal study. It is possible that the wide range observed in the dog is attributable to the trauma and anesthesia necessary in most methods used. On the other hand, man may not be capable of such a wide variation at rest because of the absence of a "reserve" apparently present in some animals.

SUMMARY

1. The hepatic blood flow in man has been estimated by the following procedure:

a. By means of a constant intravenous infusion, Bromsulphalein was given at such a rate that the concentration of the dye in the peripheral blood remained unchanged. Under these conditions, the rate of BSP removal by the liver was assumed to equal the infusion rate. This assumption appears to be valid, even if BSP is removed in the portal circulation by reticulo-endothelial cells outside the liver. Any activity of reticulo-endothelial cells in other extrahepatic depots presumably ceases when an equilibrium is reached between serum and cellular concentrations of the dye.

b. The concentration of BSP in peripheral venous blood was determined at regular intervals. This concentration, which was shown to equal that in arterial blood, was assumed to be equivalent, for the purpose of estimating hepatic blood flow, to the BSP level in blood reaching the liver.

c. The concentration of BSP in blood leaving the liver was determined at intervals which corresponded as closely as possible with the sampling of peripheral blood. Hepatic venous blood was obtained from a right hepatic vein by a modification of the venous catheterization technique of Cournand(8).

d. Given the rate at which BSP was removed from the blood by the liver, the concentration of BSP in blood afferent to the liver, and the concentration in blood leaving the liver, hepatic blood flow was calculated according to the "Fick principle." Since the BSP concentration was determined in blood leaving a portion of the liver and not in mixed venous blood from the whole liver, the calculated blood flow was designated *estimated hepatic blood flow* (EHBf).

2. In 23 subjects without gross hepatic disease, the EHBf varied from 1,085 to 1,845 ml. per minute per 1.73 M.² of body surface with an average of 1,497 ml. No evidence of irregular shunting or gross alternation of blood flow in the human liver was obtained, for the flow appeared to be quite constant under the conditions of our study.

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THE ABSORPTION AND EXCRETION OF STREPTOMYCIN IN HUMAN CHRONIC TYPHOID CARRIERS

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Streptomycin,¹ an antibiotic agent produced by the growth of *Actinomyces griseus*, has been shown to inhibit the growth of gram negative bacilli both *in vitro* (1 to 5) and in animals (2, 3, 6 to 9). Preliminary data on the absorption and excretion of this drug were obtained in a patient suffering from acute bacterial endocarditis due to *Pseudomonas aeruginosa*, the first human patient treated with streptomycin (10). The data indicated that it was possible to attain levels of the drug in the blood in the range of sensitivity of gram-negative bacilli of this antibiotic agent and justified the performance of more detailed studies of absorption and excretion of streptomycin.

Chronic typhoid carriers were selected as subjects for this study for several reasons; they were as relatively "normal" individuals as could be found in the population of the hospital and, in addition, provided an opportunity to study the effect of streptomycin on the typhoid carrier state.

METHOD OF STUDY

Four male chronic typhoid carriers, under the jurisdiction of the Department of Health of the City of New York, were admitted to the First Medical Division of Bellevue Hospital. These individuals were known to have had consistently positive cultures of typhoid bacilli in their stools without exception for at least 2 years. Following admission, a medical history was obtained and a physical examination was performed. The following control measurements in relation to this study were made: complete blood count, urinalysis, erythrocyte sedimentation rate (Westgren method), blood urea nitrogen (11), cephalin flocculation test (12), icteric index, and electrocardiogram. In addition to the tests performed in relation to drug absorption and excretion, the following measurements were made in relation to the typhoid carrier state. These included urine and stool cultures on MacConkey medium, Bacto S. S. Agar, and Wilson-Blair medium. The typhoid bacilli were typed according to the

bacteriophage method of Craigie and Yen (13). The urine cultures were performed prior to therapy and repeated twice on successive days. Stool cultures were performed prior to therapy and daily thereafter, the last specimen being taken 15 hours after therapy was discontinued. The sensitivity of the strains of typhoid bacilli were determined by the agar plate method.

An intracutaneous skin test on the flexor aspect of the right forearm was performed with 0.1 ml. of 1:10 dilution of streptomycin. Twenty minutes later the tests were read and no reactions were noted.

Prior to the administration of the drug the patients were instructed to empty their bladders. A specimen of the urine was taken for urinalysis, the remainder being discarded. Thereafter, for the duration of the study, a record was kept of the intake and output of fluids in each patient.

A dose of 75,000 units of streptomycin in 5 ml. of solution was then injected intramuscularly. The time periods indicated in all the tables of this study denote the time elapsed following the first injection. The hour when the first injection was made is considered as the zero hour in the study. One hour later a blood specimen was taken for a determination of the concentration of streptomycin. The subjects were instructed to empty their bladders, the volume of urine was measured and recorded, and a specimen of the urine was taken for streptomycin assay. Similar specimens were taken and measurements made at the end of the second and third hours after the first injection. At this latter time, the patients received another 75,000 units of streptomycin intramuscularly, blood and urine specimens being collected before the injections were given. Thereafter, during this phase of the study, each patient received 75,000 units of streptomycin intramuscularly every 3 hours until a total of 600,000 units had been given in 8 injections.

The concentration of streptomycin in the blood and urine specimens are indicated in Tables I to IV. When the time of injection coincided with the time of the taking of the specimen, samples were obtained before the injections were given.

Twenty-four hours after the onset of the study, the dose of streptomycin was increased to 150,000 units in 10 ml. of solution intramuscularly every 3 hours. At this dosage level a total of 1,950,000 units of streptomycin were given in 13 injections from the twenty-fifth to the sixtieth hour of the study.

Beginning at the sixty-third hour, the dosage was reduced to the original level of 75,000 units in 5 ml. intra-

¹ The streptomycin used in this study was supplied by Merck and Co., Inc., and consisted of lots numbered 4R6040 and 4R6223.

TABLE I

Blood and urine concentration and excretion of streptomycin in relation to dosage by time periods
Subject—Nádherný

Time period	Urine output	Dosage	Blood concentration	Urine concentration	Excretion
hour †	ml.	units per 3 hours	units per ml.		units
Control			1.0 to 2.0		
1	375	75,000 intramuscularly for 8 doses	1.0 to 2.0	40.0	15,000
2	100		1.0 to 2.0	80.0	8,000
3	100		1.0 to 2.0	80.0	8,000
12	1,125		1.0 to 2.5	80.0	90,000
24	1,890		1.0 to 2.0	140.0	264,000
25		150,000 intramuscularly for 13 doses	8.0		
26	200		13.0	160.0	32,000
27	450		6.0	140.0	63,000
36	800		13.0	400.0	320,000
37	200			400.0	80,000*
38 } 39b	250			200.0	50,000*
39	150		18.0		
39b	600			240.0	144,000
48	1,790		10.0	180.0	322,000
60	1,375		8.0	270.0	371,000
63		75,000 intramuscularly for 2 doses			
66					
69		None			
72	3,975		5.0	70.0	278,250
84	1,410		1.0 to 2.5	40.0	56,400
93	600		1.0 to 2.5	20.0	12,000
94	600	31,250 orally** for 8 doses	1.0 to 2.5	2.5	1,500
95	800		1.0 to 2.5	0	0
96	700		1.0 to 2.5	2.5	1,750
97	500		1.0 to 2.5	2.5	1,250
109	1,750		1.0 to 2.5	2.5	4,375
117	1,340		1.0 to 2.5	2.5	3,350
120	800	None	1.0 to 2.5	0	0
132	2,150		1.25	2.5	5,375

* These 2 excretion values are not included in the total excretion because sample 39b includes the total excretion during hours 37 to 39 (881,200 units).

** Blood levels following oral administration reported on a basis of estimated unitage.

† Hour at which blood was taken.

muscularly for 2 doses. Intramuscular administration was then discontinued. These latter changes in dosage were made necessary by the toxic manifestations of the particular lot of drug studied.

From the sixty-ninth through the ninety-third hour, no further drug was administered but specimens of blood and urine were collected as indicated in Tables I to IV.

Beginning at the ninety-fourth hour and ending at the one hundred seventeenth hour, 31,250 units per dose of streptomycin were administered orally in capsules for 8 doses.

From the one hundred twentieth to the one hundred thirty-second hour, no further drug was administered but specimens were again collected as indicated in Tables I to IV.

Each day during the course of the study the following measurements were made on each patient: fluid intake and urinary output, urinalysis, complete blood count, blood urea nitrogen, erythrocyte sedimentation rate, blood pressure, and electrocardiogram. Temperature, pulse, and

respiration were recorded every 4 hours throughout the day and night. An icteric index and a cephalin flocculation test were repeated at the end of the study.

Procedure for the assay of streptomycin in blood and urine²

The concentrations of streptomycin in the blood and urine were determined by a modification of

TABLE II
Blood and urine concentration and excretion of streptomycin in relation to dosage by time periods
 Subject—Russo

Time period	Urine output	Dosage	Blood concentration	Urine concentration	Excretion
hour †	ml.	units per 3 hrs.	units per ml.		units
Control			1.0 to 2.0		
1	350	75,000 intramuscularly for 8 doses	1.0 to 2.0	20.0	7,000
2	650		1.0 to 2.0	20.0	13,000
3	300		1.0 to 2.0	40.0	12,000
12	675		2.5 to 5.0	100.0	67,500
24	2,765		6.0	100.0	276,500
25		150,000 intramuscularly for 13 doses	18.0		
26	725		13.0	60.0	43,500
27	450		8.0	80.0	36,000
36	550		20.0	270.0	148,500
37					
38 } 39b	525			240.0	126,000*
39	400		20.0		
39b	925			150.0	138,750
48	2,670		20.0	180.0	480,600
60	1,725		20.0	240.0	414,000
63		75,000 intramuscularly for 2 doses			
66					
69		None			
72	3,475		15.0	80.0	278,000
84	3,350		5.0	20.0	67,000
93	2,580		1.25	5.0	12,900
94	600	31,250 orally** for 8 doses	1.0 to 2.5	5.0	3,000
95	650		1.0 to 2.5	3.0	1,950
96	700		1.0 to 2.5	5.0	3,500
97	500		1.0 to 2.5	3.0	1,500
109	2,600		1.0 to 2.5	3.0	7,800
117	2,190		1.0 to 2.5	3.0	6,570
120	1,250	None	<1.25	3.0	3,750
132	1,220		<1.25	8.0	9,760

* This excretion value is not included in the total excretion because sample 39b includes the total excretion during hours 37 to 39 (138,750 units).

** Blood levels following oral administration reported on a basis of estimated unitage.

† Hour at which blood was taken.

the Foster-Woodruff assay method (14) for streptothricin. In order to increase the sensitivity of the method, all blood samples were hemolyzed by the addition of weighed amounts (0.5 to 1.0 mgm.) of saponin. This prevented the red cells in the blood samples from settling at the base of the penicylinders, thereby facilitating diffusion of the drug through the agar.

The concentration of streptomycin in blood was determined from a standard curve of reference for each blood assay. Dilutions of the drug standard (5, 10, 20, 40, 60, 80, 100 units per ml.) were made in normal hemolyzed blood, with the latter as diluent. The diameters of the resulting zones of cleared area were plotted as ordinates against the concentrations of streptomycin as abscissae.

Test bloods were diluted when necessary with normal hemolyzed blood to contain approximately 20 to 60 units per ml., otherwise the sample was assayed undiluted. Dilutions of the test sample and each standard level were run in duplicate. The concentration of streptomycin in a test sample was obtained by determining from the standard curve the drug concentration corresponding to the diameter of the zone of inhibition and correcting for the dilution.

Distilled water replaced whole hemolyzed blood as the diluent in the assay of the urine specimens.

In the preparation of agar plates for the assay, 0.1 ml. of a standardized *B. subtilis*³ inoculum was added to each 100 ml. of molten (45 to 50° C.)

³ Supplied by Microbiological Laboratories, Merck & Co., Inc.

F.D.A. agar. By means of a calibrated wide mouth pipette, 13 ml. of the seeded agar was delivered into each petri dish. After cooling and solidification of the agar, beveled glass penicylinders were warmed in a Bunsen flame and placed upon the agar surface resulting in an effective seal between the glass cylinder and the agar. The cylinders were then filled with the test samples. For the assay of blood specimens both the standard and the test samples were placed in the icebox overnight to insure complete diffusion of the drug through the agar plate but this was not necessary in the case of urine specimens. The plates were then incubated at 30° C. for 16 to 18 hours. The diameters of the cleared areas were then recorded in millimeters.

Absorption and excretion of streptomycin

Individual observations at various time periods in urine output, blood concentration, urine concentration and excretion of streptomycin in the 4 subjects studied are detailed in Tables I to IV. In these tables it will be noted that the concentration of streptomycin in the blood resulting from a dose of 75,000 units intramuscularly every 3 hours are very low and are at the same level as the control blood specimens. They are also in approximately the same range obtainable as a result of oral administration of 31,250 units every 3 hours. These levels are so low that they cannot accurately be determined by the method used, but it is obvious that they are below the expected range of therapeutic effectiveness of the drug against most of

TABLE III

Blood and urine concentration and excretion of streptomycin in relation to dosage by time periods
Subject—Schwaid

Time period	Urine output	Dosage	Blood concentration	Urine concentration	Excretion
hour †	ml.	units per 3 hrs.	units per ml.		units
Control			1.0 to 2.0		
1	500	75,000 intramuscularly for 8 doses	2.5	20.0	10,000
2	350		1.0 to 2.0	70.0	24,500
3	400		1.0 to 2.0	80.0	32,000
12	1,325		2.5 to 5.0	100.0	132,500
24	2,540		2.5	100.0	254,000
25		150,000 intramuscularly for 13 doses	18.0		
26	150		20.0	140.0	21,000
27	175		18.0	100.0	17,500
36	640		13.0	320.0	204,000
37					
38 } 39b	125			320.0	40,000*
39	200		18.0		
39b	325			200.0	65,000
48	1,615		8.0	200.0	323,000
60	450		10.0	400.0	180,000
63		75,000 intramuscularly for 2 doses			
66					
69		None			
72	1,075		8.0	60.0	64,500
84	1,825		5.0	90.0	164,250
93	1,400		2.5	15.0	21,000
94	700	31,250 orally** for 8 doses	1.0 to 2.5	8.0	5,600
95	300		1.0 to 2.5	20.0	6,000
96	200		1.0 to 2.5	10.0	2,000
97	200		1.0 to 2.5	10.0	2,000
102	1,750		2.5	5.0	5,250
117	1,490		2.5 to 5.0	5.0	7,450
120	250	None	1.25 to 2.5	8.0	2,000
132	740		<1.25	15.0	11,900

TABLE IV
Blood and urine concentration and excretion of streptomycin in relation to dosage by time periods
Subject—Weiss

Time period	Urine output	Dosage	Blood concentration	Urine concentration	Excretion
hour †	ml.	units per 3 hrs.	units per ml.		units
Control			1.0 to 2.0		
1	325	75,000 intramuscularly for 8 doses	1.0 to 2.0	20.0	6,500
2	75		1.0 to 2.0	150.0	11,250
3	200		1.0 to 2.0	70.0	14,000
12	660		1.0 to 2.5	100.0	66,000
24	2,195		5.0	80.0	175,600
25		150,000 intramuscularly for 13 doses	5.0		
26	300		13.0	40.0	12,000
27	400		10.0	80.0	32,000
36	500		8.0	280.0	140,000
37	50			400.0	20,000*
38 } 39b	225			120.0	27,000*
39	350		13.0		
39b	625			120.0	75,000
48	2,015		10.0	200.0	403,000
60	355		6.0	200.0	71,000
63		75,000 intramuscularly for 2 doses			
66					
69		None			
72	2,695		5.0	70.0	188,650
84	2,125		1.25	20.0	42,500
93	1,500		2.5	10.0	15,000
94	700	31,250 orally** for 8 doses	1.25	0.0	0
95	400		1.25	2.5	1,000
96	400		1.0 to 2.5	2.5	1,000
97	350		1.0 to 2.5	2.5	875
109	1,250		1.0 to 2.5	2.5	3,125
117	1,660		1.0 to 2.5	0.0	0
120	650	None	1.0 to 2.5	0.0	0
132	490		<1.25	2.5	1,225

* These 2 excretion values are not included in the total excretion because sample 39b includes the total excretion during hours 37 to 39 (75,000 units).

** Blood levels following oral administration reported on the basis of estimated unitage.

† Hour at which blood was taken.

the microorganisms which have thus far been tested *in vitro* (3). However, the number of units excreted in the interval during intramuscular administration of 75,000 units every 3 hours is obviously much greater than the amount excreted as a result of the oral administration. This would indicate that very small amounts of the drug are absorbed when administered orally and that when somewhat larger amounts are absorbed, as in the intramuscular dosage of 75,000 units every 3 hours, the drug is excreted in the urine so rapidly that no significant blood level is attained.

In the second phase of the experiment, after increasing the dose to 150,000 units intramuscularly every 3 hours, the concentrations of the drug in the blood rose sharply into the range of thera-

peutic effectiveness and were maintained at that level despite excessive intake and output of fluid. Therefore, such levels can reasonably be expected to be attained with this dosage of the drug.

It is further evident from the study of Tables I to IV that even though the dosage of the drug was halved at the sixty-third hour and discontinued at the sixty-sixth hour, a measurable amount of the drug was still present in the blood until the seventy-second hour, and that excretion in the urine continued through the ninety-third hour.

The data in Table V are summarized in protracted time periods. The percentage excretion of the drug is relatively constant during the first 2 periods regardless of the increase in dosage dur-

ing the second period. However, during the third period in 2 of the 4 patients (S and W), when the urinary output dropped because of the toxic manifestations of the drug, there was a decrease in the output of the drug.

During the fourth period, following discontinuation of the drug administration at which time the output of urine increased in 3 of the 4 patients (R, S, and W), some of the retained drug was excreted. The small amount of drug absorbed

when administered by mouth is reemphasized in Table V.

In Tables VI to IX, pertinent laboratory observations are summarized. This lot of drug produced a leukocytosis with an increase in polymorphonuclear leukocytes, an increase in the sedimentation rate and an increase in the formed elements in the urine. There was a concomitant decrease in the urinary output in 2 patients (S and W). Examination of the urine approxi-

TABLE V
Dosage, excretion, and cumulative excretion of streptomycin by time periods

Time periods	Streptomycin Dosage *	Fluid Intake	Urine output	Streptomycin excretion		
				Amount in each period	Percentage in each period	Cumulative percentage
Subject—N						
<i>hours</i>	<i>units</i>	<i>ml.</i>	<i>ml.</i>	<i>units</i>		
	I.M.					
0 to 24	600,000	7,375	3,590	385,600	64.3	64.3
25 to 48	1,200,000	6,640	4,140	881,200	73.4	70.4
49 to 72	900,000	7,840	5,350	649,500	72.1	71.0
73 to 93	000,000	3,665	2,010	68,400		73.5
	P.O.					
94 to 117	250,000	7,160	5,690	12,225	4.9	67.7
118 to 132	000,000	2,000	2,950	5,375	2.2	67.9
Subject—R						
<i>hours</i>	<i>units</i>	<i>ml.</i>	<i>ml.</i>	<i>units</i>		
	I.M.					
0 to 24	600,000	8,335	4,740	376,000	62.7	62.7
25 to 48	1,200,000	8,920	5,320	847,350	70.6	68.0
49 to 72	900,000	9,580	5,200	692,000	76.8	70.9
73 to 93	000,000	3,575	5,930	79,900		73.9
	P.O.					
94 to 117	250,000	7,855	7,240	24,320	9.7	68.5
118 to 132	000,000	2,500	2,470	13,510	5.4	68.8
Subject—S						
<i>hours</i>	<i>units</i>	<i>ml.</i>	<i>ml.</i>	<i>units</i>		
	I.M.					
0 to 24	600,000	9,195	5,115	453,000	75.5	75.5
25 to 48	1,200,000	4,920	3,055	631,300	52.6	60.2
49 to 72	900,000	7,250	1,525	244,500	27.1	49.2
73 to 93	000,000	3,015	3,225	185,250		56.0
	P.O.					
94 to 117	250,000	3,215	4,640	31,800	12.7	52.4
118 to 132	000,000	1,000	990	13,100	5.3	52.9
Subject—W						
<i>hours</i>	<i>units</i>	<i>ml.</i>	<i>ml.</i>	<i>units</i>		
	I.M.					
0 to 24	600,000	5,320	3,455	273,350	45.5	45.5
25 to 48	1,200,000	9,220	3,940	662,000	55.2	51.9
49 to 72	900,000	8,050	3,050	259,650	28.8	44.3
73 to 93	000,000	4,015	3,625	57,500		49.4
	P.O.					
94 to 117	250,000	4,575	4,760	6,000	2.4	47.4
118 to 132	000,000	2,000	1,140	1,225	0.1	47.6

* A total of 2,950,000 units were administered to each subject, the first 2,500,000 intramuscularly and the last 250,000 by mouth.

TABLE VI
Pertinent laboratory observations
Subject—Nadherry

	Blood counts								Urinalyses								Erythrocyte sedimentation rate†	Blood urea nitrogen	Cephalin flocculation	Ic- teric index	Blood pressure			
	Hgb.	R.B.C.	W.B.C.	P	L	M	E	B	Sp. Gr.	Reac- tion	Albu- min	Glu- cose	Bile	R.B.C.		W.B.C.						Casts††		
														C*	N.C.**	C*						N.C.**	C*	N.C.**
hour	0	14.0	5.14	11,100	50	39	6	4	1	1.022	Acid	0	0	0	0	0	0	0	0	0	10	12	Nega- tive	150/88
12	14.5			9,100	68	28	0	4	0	1.006	Acid	+	0	0	0	0	Rare	0	0	0	5	13		120/70
39	14.5			8,900	68	24	0	8	0	1.008	Acid	+	0	0	0	0	Rare	0	0	0	7	11		110/70
48				13,900	83	13	1	3	0	1.008	Acid	+	0	0	0	0	1 to 2	5 to 10	0	0	11	13		
60	15.5			11,200	75	23	0	2	0	1.008	Acid	+	0	0	0	0	Rare	Rare	0	0	11	13		120/80
84				9,650	74	16	6	4	0	1.010	Acid	0	0	0	0	0	Rare	Rare	0	0	18	16		130/80
120	13.5			8,600	80	16	0	4	0	1.008	Acid	0	0	0	Occa- sional {2 clumps 0	0	Rare	0	0	0	33	14	Nega- tive	144/90
132										1.028	Acid	+	0	0	0 to 3	0	0	0	0	0	40			
231	13.0									1.016	Acid	+	0	0	0	0	Occa- sional	Occa- sional	0	0				125/80

* Centrifuged.

** Not centrifuged.

† Westgren method.

†† G = granular, H = hyaline, W = white blood cell, and R = red blood cell cast.

TABLE VII
Pertinent laboratory observations
Subject—Russo

Blood counts										Urinalyses										Erythrocyte sedimentation rate†	Blood urea nitrogen	Cephalin flocculation	Icteric index	Blood pressure
Hct.	H.B.C.	W.B.C.	P	L	M	E	B	Sp. Gr.	Reaction	Albumin	Glucose	Bile	R.B.C.		W.B.C.		Casts††							
													C*	N.C.**	C*	N.C.**	C*	N.C.**						
13.0	4.46	7,250	62	36	2	0	0	1.024	Acid	+	0	0	0	0	0	0	0	0	0	10	9	Negative	5	150/90
15.0		8,550	81	15	1	0	0	1.008	Acid	0	0	0	0	0	0	0	Rare	0	0	12	11			130/80
21		11,320	82	15	2	0	1	1.008	Acid	0	0	0	0	0	0	0	0	0	0					
27		9,250	82	15	1	2	0	1.005	Acid	++	0	0	0	7	0	0	(Many clumps)	(0 to 2 clumps)	(Moderate G, W and R)	28	15			120/80
49		11,300	77	19	4	0	0	1.010	Acid	++	0	0	0	0	0	0	Occasional	Occasional	(Occasional G, H and R)	41	14			122/80
60	13.1	9,400	78	21	0	0	1	1.002	Acid	++	0	0	0	1 to 2	1 to 2	(Numerous)	(Numerous)	(Numerous W, occasional G, rare R)	52	15				115/85
84		6,950	62	30	6	1	1	1.004	Acid	++	0	0	0	Occasional	Rare	0	0	0	(Rare H, G and R)	21				
94								1.002	Acid	++	0	0	0	2 to 4 clumps	0 to 2	Occasional	0	0	(Occasional H and G)	56	16	Negative	8	140/90
120	13.0	5,750	78	18	2	2	0	1.006	Acid	++	0	0	0	Masses	5 to 12	0	0	0	(Rare H, G, R and W)	49				
142								1.012	Acid	+++	0	0	0	0	0	0	0	0	(Rare G and H)					155/90
201	12.6							1.002	Neutral	++	0	0	0	0	0	Rare	0 to 1							

* Centrifuged.

** Not centrifuged.

† Western method.

†† G = granular, H = hyaline, W = white blood cell, and R = red blood cell cast.

TABLE VIII
Pertinent laboratory observations
Subject—Schwaid

Blood counts										Urinalyses										Erythrocyte sedimentation rate†	Blood urea nitrogen	Cephalin flocculation	Ic-teric index	Blood pressure																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
Hgb.	R.B.C.	W.B.C.	P	L	M	E	B	Sp. Gr.	Reaction	Albumin	Glucose	Bile	R.B.C.		W.B.C.		Casts††																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
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* Centrifuged.

** Not centrifuged.

† Westgren method.

†† G = granular, H = hyaline, W = white blood cell, and R = red blood cell cast.

STREPTOMYCIN IN HUMAN CHRONIC TYPHOID CARRIERS

TABLE IX
Pertinent laboratory observations
Subject—Weiss

Blood counts										Urinalyses										Erythrocyte sedimentation rate†	Blood urea nitrogen	Cephalin flocculation	Ic-teric index	Blood pressure
Hgb.	R.B.C.	W.B.C.	P	L	M	E	B	Sp. Gr.	Reaction	Albumin	Glucose	Bile	R.B.C.		W.B.C.		Casts††							
													C*	N.C.**	C*	N.C.**	C*	N.C.**						
11.5	5.30	10,520	61	33	6	0	0	1.023	Acid	+	0	0	0	0	0	0	0	0	0	7	13	Negative	4	130/90
12	11.5	8,310	88	12	0	0	0	1.018	Acid	++	0	0	0	0	0	Rare	Occasional	Rare	G	5	10			120/60
39	15.0	9,100	72	22	2	4	0	1.005	Acid	+	0	0	0	0	0	Occasional	Occasional	G and H	G and H	13	11			118/68
18		13,900	76	15	8	1	0	1.002		0	0	0	0	0	0	1 to 3	Rare	0	0	0	7			128/80
60	17.5	12,100	68	29	1	2	0	1.002	Acid	++	+	0	0	0	0	0	Rare	Occasional	Occasional H and G	23	16			
81		9,800	70	25	4	0	1	1.004	Acid	++	0	0	0	0	0	Rare	Rare	0	0	31	12			120/80
120	12.2	6,500	70	28	1	1	0		Acid	++	++	0	Rare	0	0	0	Occasional	Rare	H	15	13	Negative	12	120/70
112								1.012	Acid	+	0	0	0	0	0	Occasional	Rare	0	0	31				118/80
211								1.012	Acid	+	0	0	0	0	0	Occasional	Rare	0	0					

* Centrifuged.

** Not centrifuged.

† Westgren method.

†† G = granular, H = hyaline, W = white blood cell, and R = red blood cell cast.

mately 4 days following the completion of the experiment at the two hundred thirty-first hour showed a decrease in the abnormal findings in the urine.

The patients also developed fever and signs of inflammation at the sites of injection. From more recent experience in the use of this drug (16) the toxic manifestations demonstrated by the 2 lots of drugs studied would be considered atypical and have not resulted from therapy with subsequent lots of streptomycin. The only change noted in the cardiovascular system was a depression in the systolic and diastolic blood pressure which became manifest very early in the course of drug administration as indicated by the blood pressure measurements at the twelfth hour in Tables VI to VIII. The daily electrocardiograms showed no significant changes.

The data in 2 recent publications (17, 18), utilizing the same method of assay described in this paper, could not be compared with these results since the minimum period for which data were presented in those studies was 24 hours.

Typhoid carrier study

The stool cultures taken prior to the administration of the drug were all strongly positive for *B. typhosus*. The typhoid bacilli isolated from patient R proved to be type E, and that from patient W, type A. The typhoid bacilli isolated from the other 2 patients did not type. The sensitivity of the typhoid bacilli to streptomycin ranged between 10 and 20 units per ml. (S = 10 units per ml.; N, R, and W = 20 units per ml.).

There were no significant changes in the number of typhoid bacilli in the stools of any of the patients during the intramuscular administration of the drug. Stool specimens from 2 of 3 patients (from patient S and W but not from patient R), obtained at the end of the 24-hour period of oral administration of 31,250 units of the drug every 3 hours, were negative for typhoid bacilli but were positive again 12 hours later.

The urine cultures were negative prior to therapy and remained so.

SUMMARY AND CONCLUSIONS

1. Absorption and excretion of streptomycin following intramuscular and oral administration

were studied in 4 human typhoid carriers. Streptomycin was administered every 3 hours in a dosage of 75,000 and 150,000 units intramuscularly and 31,250 units orally.

2. Following intramuscular administration of streptomycin approximately 60 to 70 per cent of the drug was excreted in the urine during a 24-hour period. Appreciable amounts of the drug were not absorbed from the gastrointestinal tract following repeated administration of capsules containing 31,250 units.

3. Blood concentrations of streptomycin following administration of 75,000 units intramuscularly every 3 hours ranged from 0 to 6 units per ml. and when the dose was increased to 150,000 units every 3 hours, the level rose to 5 to 20 units per ml.

4. Certain toxic manifestations unique for these particular lots of drug were noted.

5. At a dosage level of 75,000 or 150,000 units intramuscularly every 3 hours, no significant bacteriostatic effect on the typhoid bacilli in the stool was obtained. Following oral administration of 31,250 units every 3 hours, a definite transitory bacteriostatic effect on the typhoid bacilli in the stool was obtained in 2 of 3 patients.

The authors wish to express their appreciation to Dr. Harry J. Robinson for his most helpful assistance during the course of this study.

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AUTHOR INDEX

A

- Abbott, William E. See MEYER, JOSEPH, HIRSHFELD, and ABBOTT 579
- Abelson, Neva M. See DIAMOND and ABELSON 122
- Adams, M. A. See TAYLOR, DAVIDSON, TAGNON, ADAMS, MACDONALD, and MINOT 698
- Albright, F. See REIFENSTEIN, FORBES, ALBRIGHT, DONALDSON, and CARROLL 416
- Aliminosa, Lucy. See SMITH, FINKELSTEIN, ALIMINOSA, CRAWFORD, and GRABER 388
- Allen, Howard E. See FINLAND, PETERSON, ALLEN, SAMPER, and BARNES 451
- See FINLAND, PETERSON, ALLEN, SAMPER, and BARNES 458
- Altschule, M. D., Freedberg, A. S., and McManus, M. J. Circulation and respiration during an episode of chill and fever in man 878
- See FRANK, ALTSCHULE, and ZAMCHECK 54
- Annegers, J. A. See DRILL, ANNEGERS, SNAPP, and IVY 97
- Appelbaum, Emanuel. See FALK and APPELBAUM 742
- Armstrong, Charles D. See BLOOMFIELD, ARMSTRONG, and KIRBY 251
- Armstrong, S. H., Jr. See THORN, ARMSTRONG, DAVENPORT, WOODRUFF, and TYLER 802
- Ashworth, J. N. See SCATCHARD, STRONG, HUGHES, ASHWORTH, and SPARROW 671
- Aub, J. C., Brues, A. M., Kety, S. S., Nathanson, I. T., Nutt, A. L., Pope, A., and Zamecnik, P. C. The toxic factors in experimental traumatic shock. IV. The effects of the intravenous injection of the effusion from ischemic muscle 848
- See NATHANSON, NUTT, POPE, ZAMECNIK, AUB, BRUES, and KETY 829
- See BRUES, COHN, KETY, NATHANSON, NUTT, TIBBETTS, ZAMECNIK, and AUB 838
- See KETY, NATHANSON, NUTT, POPE, ZAMECNIK, AUB, and BRUES 842
- See ZAMECNIK, AUB, BRUES, KETY, NATHANSON, NUTT, and POPE 853
- See POPE, ZAMECNIK, AUB, BRUES, DUBOS, NATHANSON, and NUTT 859

B

- Baldwin, E. deF. See COURNAND, RILEY, BREED, BALDWIN, and RICHARDS 106
- Barnes, Mildred W. See FINLAND, BARNES, and SAMPER 192
- See FINLAND, PETERSON, ALLEN, SAMPER, and BARNES 451
- See FINLAND, PETERSON, ALLEN, SAMPER, and BARNES 458
- See FINLAND, PETERSON, and BARNES 474
- See FINLAND, SAMPER, and BARNES 483
- See FINLAND and BARNES 490
- See FINLAND, SAMPER, and BARNES 497

- Beckman, William W. See EMERSON and BECKMAN 564
- Beecher, Henry K. See BURNETT, BLAND, and BEECHER 694
- Bigelow, Nolton, Harrison, Irving, Goodell, Helen, and Wolff, Harold G. Studies on pain: quantitative measurements of two pain sensations of the skin, with reference to the nature of the "hyperalgesia of peripheral neuritis" 503
- Bing, R. J., Thomas, C. B., and Waples, E. C. The circulation in experimental neurogenic hypertension 513
- Bland, Edward F. See BURNETT, BLAND, and BEECHER 694
- Bloomfield, Arthur L., Armstrong, Charles D., and Kirby, William M. M. The treatment of subacute bacterial endocarditis with penicillin 251
- Blum, Harold F. The solar heat load: its relationship to total heat load and its relative importance in the design of clothing 712
- Boothby, Walter M. See PETERS, HORTON, and BOOTHBY 611
- Bradley, G. P. See BRADLEY, INGELFINGER, BRADLEY, and CURRY 890
- Bradley, Stanley E., Chasis, Herbert, Goldring, William, and Smith, Homer W. Hemodynamic alterations in normotensive and hypertensive subjects during the pyrogenic reaction 749
- , Ingelfinger, F. J., Bradley, G. P., and Curry, J. J. The estimation of hepatic blood flow in man 890
- Brannon, E. S., Merrill, A. J., Warren, J. V., and Stead, E. A., Jr. The cardiac output in patients with chronic anemia as measured by the technique of right atrial catheterization 332
- See STEAD, WARREN, MERRILL, and BRANNON 326
- See WARREN, BRANNON, STEAD, and MERRILL 337
- Breed, E. S. See COURNAND, RILEY, BREED, BALDWIN, and RICHARDS 106
- Breslow, Lester. Epidemic of acute respiratory disease associated with atypical pneumonia 775
- Bridges, William C. See DEXTER, HAYNES, and BRIDGES 62
- Brown, Philip N. See FRANCIS, SALK, PEARSON, and BROWN 536
- See SALK, PEARSON, BROWN, and FRANCIS 547
- Brues, A. M., Cohn, W. E., Kety, S. S., Nathanson, I. T., Nutt, A. L., Tibbetts, D. M., Zamecnik, P. C., and Aub, J. C. The toxic factors in experimental traumatic shock. II. Studies on electrolyte and water balance in shock 838
- See NATHANSON, NUTT, POPE, ZAMECNIK, AUB, BRUES, and KETY 829
- See KETY, NATHANSON, NUTT, POPE, ZAMECNIK, AUB, and BRUES 842

- See AUB, BRUES, KETY, NATHANSON, NUTT, POPE, and ZAMECNIK 848
- See ZAMECNIK, AUB, BRUES, KETY, NATHANSON, NUTT, and POPE 853
- See POPE, ZAMECNIK, AUB, BRUES, DUBOS, NATHANSON, and NUTT 859
- Burnett, Charles H., Bland, Edward F., and Beecher, Henry K. Electrocardiograms in traumatic shock in man 694
- Burrows, Belton A. See GROSSMAN, SAPPINGTON, BURROWS, LAVIETES, and PETERS 523
- Buschke, W. H. See CARTWRIGHT, WINTROBE, BUSCHKE, FOLLIS, SUKSTA, and HUMPHREYS 268

C

- Cambier, Margaret J. See TILLET, MCCORMACK, and CAMBIER 589
- See TILLET, MCCORMACK, and CAMBIER 595
- Cameron, James W., and Diamond, Louis K. Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXIX. Serum albumin as a diluent for anti-Rh typing reagents 793
- Carroll, E. See REIFENSTEIN, FORBES, ALBRIGHT, DONALDSON, and CARROLL 416
- Cartwright, G. E., Wintrobe, M. M., Buschke, W. H., Follis, R. H., Jr., Suksta, A., and Humphreys, S. Anemia, hypoproteinemia, and cataracts in swine fed casein hydrolysate or zein. Comparison with pyridoxine-deficiency anemia 268
- Cathcart, Richard T. See RUTSTEIN, STEBBINS, CATHCART, and HARVEY 898
- Chasis, Herbert, Redish, Jules, Goldring, William, Ranges, Hilmert A., and Smith, Homer W. The use of sodium p-aminohippurate for the functional evaluation of the human kidney 583
- See BRADLEY, CHASIS, GOLDRING, and SMITH 749
- Clarke, Delphine. See RALLI, ROBSON, CLARKE, and HOAGLAND 316
- Cohn, W. E. See BRUES, COHN, KETY, NATHANSON, NUTT, TIBBETTS, ZAMECNIK, and AUB 838
- Commission on Acute Respiratory Diseases. An experimental attempt to transmit primary atypical pneumonia in human volunteers 175
- Cope, Oliver. See RAWSON, MOORE, PEACOCK, MEANS, COPE, and RIDDELL 869
- Cournand, A., Riley, R. L., Breed, E. S., Baldwin, E. deF., and Richards, D. W., Jr. Measurement of cardiac output in man using the technique of catheterization of the right auricle or ventricle 106
- Crawford, Betty. See SMITH, FINKELSTEIN, ALMINOSA, CRAWFORD, and GRABER 388
- Culbertson, James T. See ROSE, CULBERTSON, and LIFMAN 532
- Curnen, Edward C., Mirick, George S., Ziegler, James E., Jr., Thomas, Lewis, and Horsfall, Frank L., Jr. Studies on primary atypical pneumonia. I. Clinical features and results of laboratory investigations 209
- See THOMAS, MIRICK, CURNEN, ZIEGLER, and HORSFALL 227
- Curry, J. J. See BRADLEY, INGELFINGER, BRADLEY, and CURRY 890
- Curtis, Raymond M. See LONGSWORTH, CURTIS, and PEMBROKE 46

D

- Davidson, C. S. See TAYLOR, DAVIDSON, TAGNON, ADAMS, MACDONALD, and MINOT 698
- See MINOT, DAVIDSON, LEWIS, TAGNON, and TAYLOR 704
- Davenport, V. D. See THORN, ARMSTRONG, DAVENPORT, WOODRUFF, and TYLER 802
- Dekaneas, Demetre. See PROGER, DEKANEAS, and SCHMIDT 864
- Dexter, Lewis, Haynes, Florence W., and Bridges, William C. The renal humoral pressor mechanism in man. I. Preparation and assay of human renin, human hypertensinogen, and hypertensin 62
- See QUINBY, DEXTER, SANDMEYER, and HAYNES 69
- See HAYNES and DEXTER 75
- See HAYNES and DEXTER 78
- Diamond, Louis K., and Abelson, Neva M. The importance of Rh inhibitor substance in anti-Rh serums 122
- See CAMERON and DIAMOND 793
- Dole, Vincent P., and Emerson, Kendall, Jr. Electrophoretic changes in the plasma protein patterns of patients with relapsing malaria 644
- Watson, Robert F., and Rothbard, Sidney. Electrophoretic changes in the serum protein patterns of patients with scarlet fever and rheumatic fever 648
- Donaldson, E. See REIFENSTEIN, FORBES, ALBRIGHT, DONALDSON, and CARROLL 416
- Dowling, Harry F., Mayer, Ruth L., Sweet, Lewis K., and Dumoff-Stanley, Edith. A study of the agglutinin response in patients with meningococcic meningitis 160
- Drill, Victor A., Annegers, J. A., Snapp, E. F., and Ivy, A. C. Effect of biliary fistula on bromsulphalein retention, serum phosphatase, and bile phosphatase 97
- Dubos, R. J. See POPE, ZAMECNIK, AUB, BRUES, DUBOS, NATHANSON, and NUTT 859
- Dumoff-Stanley, Edith. See DOWLING, MAYER, SWEET, and DUMOFF-STANLEY 160
- Durlacher, Stanley H. See MAN, KATZ, DURLACHER, and PIERCE 624

E

- Easton, Monroe D. See MURPHY, EASTON, and VAN HERTEN 741

Ellerbrook, Lester D. See LIPPINCOTT, ELLERBROOK, HESSELBROCK, GORDON, GOTTLIEB, and MARBLE 616

Emerson, Kendall, Jr., and Beckman, William W. Calcium metabolism in nephrosis. I. A description of an abnormality in calcium metabolism in children with nephrosis 564

— See DOLE and EMERSON 644

Enders, John F. See HARTLEY, ENDERS, MUELLER, and SCHOENBACH 92

Engel, George L., Webb, Joseph P., and Ferris, Eugene B. Quantitative electroencephalographic studies of anoxia in humans; comparison with acute alcoholic intoxication and hypoglycemia 687

F

Falk, Carolyn R., and Appelbaum, Emanuel. Type specific meningococcic agglutinins: II—the relationship of titers to the course of the disease 742

Favour, Cutting B. See METCOFF, FAVOUR, and STARE 82

Feldman, Harry A., and Murphy, Franklin D. The effect of alterations in blood volume on the anemia and hypoproteinemia of human malaria 780

Ferris, Eugene B. See ENGEL, WEBB, and FERRIS 687

Fine, Jacob. See FRANK, SELIGMAN, and FINE 435

— See GOLDBERG and FINE 445

Fink, K. See RICCA, FINK, KATZIN, and WARREN 127

— See RICCA, FINK, STEADMAN, and WARREN 140

— See RICCA, FINK, and WARREN 146

— See SCHOLZ, SCHULTZ, PLEUNE, FINK, STEADMAN, and WARREN 154

Finkelstein, Norma. See SMITH, FINKELSTEIN, ALIMINOSA, CRAWFORD, and GRABER 388

Finland, Maxwell, Barnes, Mildred W., and Samper, Bernardo A. Influenza virus isolations and serological studies made in Boston during the winter of 1943–1944 192

—, Peterson, Osler L., Allen, Howard E., Samper, Bernardo A., and Barnes, Mildred W. Cold agglutinins. I. Occurrence of cold isohemagglutinins in various conditions 451

—, —, —, —, and —. Cold agglutinins. II. Cold isohemagglutinins in primary atypical pneumonia of unknown etiology with a note on the occurrence of hemolytic anemia in these cases 458

—, —, and Barnes, Mildred W. Cold agglutinins. III. Observations on certain serological and physical features of cold agglutinins in cases of primary atypical pneumonia and of hemolytic anemia 474

—, Samper, Bernardo A., and Barnes, Mildred W. Cold agglutinins. IV. Critical analysis of certain aspects of the method for determining cold isohemagglutinins 483

—, —, and Barnes, Mildred W. Cold agglutinins. V. Deterioration of cold isohemagglutinins on storage 490

—, Samper, Bernardo A., and Barnes, Mildred W. Cold agglutinins. VI. Agglutinins for an indifferent streptococcus in primary atypical pneumonia and in other conditions and their relation to cold isohemagglutinins 497

Fleischmann, Walter. See WILKINS and FLEISCHMANN 21

Fletcher, A. G., Jr., Hardy, James D., Riegel, C., and Koop, C. E. Gelatin as a plasma substitute: the effects of intravenous infusion of gelatin on cardiac output and other aspects of the circulation of normal persons, of chronically ill patients, and of normal volunteers subjected to large hemorrhage 405

Flippin, Harrison F. See REINHOLD, FLIPPIN, ZIMMERMAN, GEFTER, and RIDDLER 352

Floody, Robert J. See RUTSTEIN, THOMSON, TOLMACH, WALKER, and FLOODY 11

Follis, R. H., Jr. See CARTWRIGHT, WINTROBE, BUSCHKE, FOLLIS, SUKSTA, and HUMPHREYS 268

Forbes, A. P. See REIFENSTEIN, FORBES, ALBRIGHT, DONALDSON, and CARROLL 416

Foster, A. D., Jr. See NEUMANN, FOSTER, and ROVENSTINE 345

Francis, Thomas, Jr., Salk, Jonas E., Pearson, Harold E., and Brown, Philip N. Protective effect of vaccination against induced influenza A 536

— See SALK, PEARSON, BROWN, and FRANCIS 547

Frank, Howard A., Altschule, Mark D., and Zamcheck, Norman. Traumatic shock. IX. Pressor therapy: The effect of paredrine on the circulation in hemorrhagic shock in dogs 54

—, Seligman, Arnold M., and Fine, Jacob. Traumatic shock. X. The treatment of hemorrhagic shock irreversible to replacement of blood volume deficiency 435

Freedberg, A. S. See ALTSCHULE, FREEDBERG, and McMANUS 878

G

Gefter, William I. See REINHOLD, FLIPPIN, ZIMMERMAN, GEFTER, and RIDDLER 352

Giacomino, N. J. See MCCHESENEY and GIACOMINO 680

Gilligan, D. Rourke. Comparative studies of the chemical changes occurring in sulfonamide drugs during therapy in man 301

Gold, Harry. See MODELL and GOLD 384

Goldberg, Martha, and Fine, Jacob. Traumatic shock. XI. Intestinal absorption in hemorrhagic shock 445

Goldring, William. See CHASIS, REDISH, GOLDRING, RANGES, and SMITH 583

— See BRADLEY, CHASIS, GOLDRING, and SMITH 749

- Goodell, Helen. See BIGELOW, HARRISON, GOOD-
ELL, and WOLFF 503
- Gordon, Harry H. See LIPPINCOTT, GORDON,
HESSELBROCK, and MARBLE 362
- See LIPPINCOTT, ELLERBROOK, HESSELBROCK,
GORDON, GOTTLIEB, and MARBLE 616
- Gottlieb, Leo. See LIPPINCOTT, ELLERBROOK, HES-
SELBROCK, GORDON, GOTTLIEB, and MARBLE 616
- Graber, Martha. See SMITH, FINKELSTEIN, ALI-
MINOSA, CRAWFORD, and GRABER 388
- Grossman, Charles M. The effect of amino acids
on serum and urine creatine 380
- , Sappington, Thomas S., Burrows, Belton A.,
Lavietes, Paul H., and Peters, John P. Nitro-
gen metabolism in acute infections 523
- Guest, George M., Rapoport, S., and Roscoe, C.
The effect of salicylates on the electrolyte
structure of the blood plasma. II. The action
of therapeutic doses of sodium salicylate and of
acetylsalicylic acid in man 770
- See RAPOPORT and GUEST 759
- Guttman, Samuel A., Potter, Harry R., Hanger,
Franklin M., Moore, David B., Pierson, Paul S.,
and Moore, Dan H. Significance of cephalin-
cholesterol flocculation test in malarial fever 296

H

- Hanger, Franklin M. See MOORE, PIERSON, HANG-
ER, and MOORE 292
- See GUTTMAN, POTTER, HANGER, MOORE,
PIERSON, and MOORE 296
- Hardy, James D. See FLETCHER, HARDY, RIEGEL,
and KOOP 405
- Harrison, Irving. See BIGELOW, HARRISON, GOOD-
ELL, and WOLFF 503
- Hartley, George, Jr., Enders, John F., Mueller, J.
Howard, and Schoenbach, Emanuel B. Ab-
sence of clinical disease in spite of a high in-
cidence of carriers of group A hemolytic strepto-
cocci of a single type; failure of tyrothricin to
influence the carrier rate 92
- Harvey, Rejane M. See RUTSTEIN, STEBBINS,
CATHCART, and HARVEY 898
- Hay, Alice L. See HEGSTED, HAY, and STARE 657
- Haynes, Florence W., and Dexter, Lewis. The
renal humoral pressor mechanism in man. III.
The hypertensinase content of plasma of
control subjects and of patients with hyper-
tension and other diseases 75
- , and —. The renal humoral pressor mechanism
in man. IV. The hypertensinogen content of
the plasma of normal patients and patients with
various diseases 78
- See DEXTER, HAYNES, and BRIDGES 62
- See QUINBY, DEXTER, SANDMANN, and
HAYNES 62
- Hegsted, D. Mark, Hay, Alice L., and Stare, F. J.
Chemical, clinical, and immunological studies

- on the products of human plasma fractionation.
XXIV. Studies on the nutritive value of
human plasma fractions 657
- Hesselbrock, Wm. B. See LIPPINCOTT, GORDON,
HESSELBROCK, and MARBLE 362
- See LIPPINCOTT, ELLERBROOK, HESSELBROCK,
GORDON, GOTTLIEB, and MARBLE 616
- Hirshfeld, John W. See MEYER, JOSEPH, HIRSH-
FELD, and ABBOTT 579
- Hoagland, Charles L. See RALLI, ROBSON, CLARKE,
and HOAGLAND 316
- Homburger, F. A plasma fibrinogen increasing
factor obtained from sterile abscesses in dogs 43

- Horsfall, Frank L., Jr. See CURNEN, MIRICK,
ZIEGLER, THOMAS, and HORSFALL 209
- See THOMAS, MIRICK, CURNEN, ZIEGLER, and
HORSFALL 227
- Horton, Bayard T. See PETERS, HORTON, and
BOOTHBY 611
- Hughes, W. L., Jr. See SCATCHARD, STRONG,
HUGHES, ASHWORTH, and SPARROW 671
- Humphreys, S. See CARTWRIGHT, WINTROBE,
BUSCHKE, FOLLIS, SUKSTA, and HUMPHREYS 268

I

- Ingelfinger, F. J. See BRADLEY, INGELFINGER,
BRADLEY, and CURRY 890
- Ivy, A. C. See DRILL, ANNEGERS, SNAPP, and Ivy 97

J

- Joseph, Samuel. See MEYER, JOSEPH, HIRSHFELD,
and ABBOTT 579

K

- Kartin, Bernard L. See MAN, KARTIN, DURLACHER,
and PETERS 623
- Katzin, Leonard I., Ricca, Renato A., and Warren,
Stafford L. Effect of environmental tempera-
ture and anesthesia on the survival of tourniquet
shock in rabbits 149
- , and Warren, Stafford L. Thiamine-deficient
diet in tourniquet shock in rats 152
- See RICCA, FINK, KATZIN, and WARREN 127
- Kety, S. S., Nathanson, I. T., Nutt, A. L., Pope, A.,
Zamecnik, P. C., Aub, J. C., and Brues, A. M.
The toxic factors in experimental traumatic
shock. III. Shock accompanying metabolic ac-
tivity and loss of vascular fluid 542
- See NATHANSON, NUTT, POPE, ZAMECNIK, AUB,
BRUES, and KETY 529
- See BRUES, COHEN, KETY, NATHANSON, NUTT,
THOMPSON, ZAMECNIK, and AUB 535
- See AUB, BRUES, KETY, NATHANSON, NUTT,
POPE, and ZAMECNIK 543
- See ZAMECNIK, AUB, BRUES, KETY, NATHAN-
SON, NUTT, and POPE 533

- Kirby, William M. M. Bacteriostatic and lytic actions of penicillin on sensitive and resistant staphylococci 165
 —. Properties of a penicillin inactivator extracted from penicillin-resistant staphylococci 170
 —. See BLOOMFIELD, AMSTRONG, and KIRBY 251
 Klopp, Calvin, Young, Nelson F., and Taylor, Howard C., Jr. Probable errors in the simultaneous measurement of separate kidney functions 117
 —, —, and —. The effects of testosterone and of testosterone propionate on renal functions in man 189
 Koop, C. E. See FLETCHER, HARDY, RIEGEL, and KOOP 405

L

- Lavietes, Paul H. See GROSSMAN, SAPPINGTON, BURROWS, LAVIETES, and PETERS 523
 Lawrence, John S. See YOUNG and LAWRENCE 554
 Lewis, Jessica H. See MINOT, DAVIDSON, LEWIS, TAGNON, and TAYLOR 704
 Lipman, Miriam Olmstead. See ROSE, CULBERTSON, and LIPMAN 532
 Lippincott, Stuart W., Gordon, Harry H., Hesselbrock, Wm. B., and Marble, Alexander. Complement fixation in human malaria using an antigen prepared from the chicken parasite *Plasmodium Gallinaceum* 362
 —, Ellerbrook, Lester D., Hesselbrock, Wm. B., Gordon, Harry H., Gottlieb, Leo, and Marble, Alexander. Liver function tests in chronic relapsing vivax malaria 616
 Longworth, Lewis G., Curtis, Raymond M., and Pembroke, Richard H., Jr. The electrophoretic analysis of maternal and fetal plasmas and sera 46
 Lozner, Eugene L. See RHODE, MORALES, and LOZNER 372
 Lubschez, Rose. Studies in ascorbic acid with especial reference to the white layer. I. Description of method and comparison of ascorbic acid levels in whole blood, plasma, red cells, and white layer 573

M

- MacDonald, A. H. See TAYLOR, DAVIDSON, TAGNON, ADAMS, MACDONALD, and MINOT 698
 Man, Evelyn B., Kartin, Bernard L., Durlacher, Stanley H., and Peters, John P. The lipids of serum and liver in patients with hepatic diseases 623
 —. See RIGGS, MAN, and WINKLER 722
 —. See WINKLER, RIGGS, and MAN 732
 Marble, Alexander. See LIPPINCOTT, GORDON, HESSELBROCK, and MARBLE 362
 —. See LIPPINCOTT, ELLERBROCK, HESSELBROCK, GORDON, GOTTLIEB, and MARBLE 616

- Mayer, Ruth L. See DOWLING, MAYER, SWEET, and DUMOFF-STANLEY 160
 McChesney, E. W., and Giacomino, N. J. The treatment of experimental hypoparathyroidism in dogs 680
 McCormack, James E. See TILLET, MCCORMACK, and CAMBIER 589
 —. See TILLET, MCCORMACK, and CAMBIER 595
 McManus, M. J. See ALTSCHULE, FREEDBERG, and MCMANUS 878
 Means, J. H. See RAWSON, MOORE, PEACOCK, MEANS, COPE, and RIDDELL 869
 Meiklejohn, Gordon, Eaton, Monroe D., and van Herick, William. A clinical report on cases of primary atypical pneumonia caused by a new virus 241
 Melin, Marshall. Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXV. The preparation of anti-A isoagglutinin reagents from mixed blood of groups O and B 662
 Merrill, A. J. See STEAD, WARREN, MERRILL, and BRANNON 326
 —. See BRANNON, MERRILL, WARREN, and STEAD 332
 —. See WARREN, BRANNON, STEAD, and MERRILL 337
 Metcalf, Jack, Favour, Cutting B., and Stare, F. J. Plasma protein and hemoglobin in the protein-deficient rat. A three-dimensional study. 82
 Meyer, Frieda L., Joseph, Samuel, Hirshfeld, John W., and Abbott, William E. Metabolic alterations following thermal burns. I. Nitrogen balance in experimental burns 579
 Minot, George R., Davidson, C. S., Lewis, Jessica H., Tagnon, H. J., and Taylor, F. H. L. The coagulation defect in hemophilia: the effect, in hemophilia, of the parenteral administration of a fraction of the plasma globulins rich in fibrinogen 704
 —. See TAYLOR, DAVIDSON, TAGNON, ADAMS, MACDONALD, and MINOT 698
 Mirick, George S. See CURNEN, MIRICK, ZIEGLER, THOMAS, and HORSFALL 209
 —. See THOMAS, MIRICK, CURNEN, ZIEGLER, and HORSFALL 227
 Modell, Walter, and Gold, Harry. Comparison of the diuretic action of sodium dehydrocholate and mercupurin in man 384
 Moore, Dan H. See MOORE, PIERSON, HANGER, and MOORE 292
 —. See GUTTMAN, POTTER, HANGER, MOORE, PIERSON, and MOORE 296
 Moore, David B., Pierson, Paul S., Hanger, Franklin M., and Moore, Dan H. Mechanism of the positive cephalin-cholesterol flocculation reaction in hepatitis 292
 —. See GUTTMAN, POTTER, HANGER, MOORE, PIERSON, and MOORE 296

- Moore, Francis D. See RAWSON, MOORE, PEACOCK, MEANS, COPE, and RIDDELL 869
- Moosnick, F. B., Schleicher, E. M., and Peterson, W. E. Progressive Addisonian pernicious anemia, successfully treated with intravenous choline chloride 278
- Morales, Manuel F. See RHODE, MORALES, and LOZNER 372
- Mueller, J. Howard. See HARTLEY, ENDERS, MUELLER, and SCHOENBACH 92
- Murphy, Franklin D. See FELDMAN and MURPHY 780

N

- Nathanson, I. T., Nutt, A. L., Pope, A., Zamecnik, P. C., Aub, J. C., Brues, A. M., and Kety, S. S. The toxic factors in experimental traumatic shock. I. Physiologic effects of muscle ligation in the dog 829
- See BRUES, COHN, KETY, NATHANSON, NUTT, TIBBETTS, ZAMECNIK, and AUB 838
- See KETY, NATHANSON, NUTT, POPE, ZAMECNIK, AUB, and BRUES 842
- See AUB, BRUES, KETY, NATHANSON, NUTT, POPE, and ZAMECNIK 848
- See ZAMECNIK, AUB, BRUES, KETY, NATHANSON, NUTT, and POPE 853
- See POPE, ZAMECNIK, AUB, BRUES, DUBOS, NATHANSON, and NUTT 859
- Neumann, C., Foster, A. D., Jr., and Rovenstine, E. A. The importance of compensating vasoconstriction in unanesthetized areas in the maintenance of blood pressure during spinal anesthesia 345
- Nutt, A. L. See NATHANSON, NUTT, POPE, ZAMECNIK, AUB, BRUES, and KETY 829
- See BRUES, COHN, KETY, NATHANSON, NUTT, TIBBETTS, ZAMECNIK, and AUB 838
- See KETY, NATHANSON, NUTT, POPE, ZAMECNIK, AUB, and BRUES 842
- See AUB, BRUES, KETY, NATHANSON, NUTT, POPE, and ZAMECNIK 848
- See ZAMECNIK, AUB, BRUES, KETY, NATHANSON, NUTT, and POPE 853
- See POPE, ZAMECNIK, AUB, BRUES, DUBOS, NATHANSON, and NUTT 859

P

- Pass, I. J., Schwartz, S., and Watson, C. J. The conversion of hematin to bilirubin following intravenous administration in human subjects 283
- Peacock, Wendell. See RAWSON, MOORE, PEACOCK, MEANS, COPE, and RIDDELL 869
- Pearson, Harold E. See FRANCIS, SALE, PEARSON, and BROWN 536
- See SALE, PEARSON, BROWN, and FRANCIS 547
- Pembroke, Richard H., Jr. See LONGWORTH, CURTIS, and PEMROKE 46

- Perera, George A. The increased plasma volume in cardiac insufficiency: its correlation with right-sided failure 708
- Peters, Gustavus A., Horton, Bayard T., and Boothby, Walter M. The effect of continuous intravenous administration of histamine on basal metabolism in human beings 611
- Peters, John P. See GROSSMAN, SAPPINGTON, BURROWS, LAVIETES, and PETERS 523
- See MAN, KARTIN, DURLACHER, and PETERS 623
- Peterson, Osler L. See FINLAND, PETERSON, ALLEN, SAMPER, and BARNES 451
- See FINLAND, PETERSON, ALLEN, SAMPER, and BARNES 458
- See FINLAND, PETERSON, and BARNES 474
- Peterson, W. E. See MOOSNICK, SCHLEICHER, and PETERSON 278
- Pierson, Paul S. See MOORE, PIERSON, HANGER, and MOORE 292
- See GUTTMAN, POTTER, HANGER, MOORE, PIERSON, and MOORE 296
- Pleune, F. Gordon. See SCHÖLZ, SCHULTZ, PLEUNE, FINK, STEADMAN, and WARREN 154
- Pope, A., Zamecnik, P. C., Aub, J. C., Brues, A. M., Dubos, R. J., Nathanson, I. T., and Nutt, A. L. The toxic factors in experimental traumatic shock. VI. The bacterial flora of ischemic muscle exudates in relation to their toxicity 859
- See NATHANSON, NUTT, POPE, ZAMECNIK, AUB, BRUES, and KETY 829
- See KETY, NATHANSON, NUTT, POPE, ZAMECNIK, AUB, and BRUES 842
- See AUB, BRUES, KETY, NATHANSON, NUTT, POPE, and ZAMECNIK 848
- See ZAMECNIK, AUB, BRUES, KETY, NATHANSON, NUTT, and POPE 853
- Potter, Harry R. See GUTTMAN, POTTER, HANGER, MOORE, PIERSON, and MOORE 296
- Proger, Samuel, Dekaneas, Demetre, and Schmidt, Gerhard. Some observations on the effect of injected cytochrome C in animals 864

Q

- Quinby, William C., Dexter, Lewis, Sandmeyer, John A., and Haynes, Florence W. The renal humoral pressor mechanism in man. II. The effect of transitory complete constriction of the human renal artery on blood pressure and on the concentration of renin, hypertensinogen, and hypertensinase of renal arterial and venous blood, with animal observations 69

R

- Ralli, Elaine P., Robson, James S., Clarke, Delphine, and Headland, Charles L. Factors in bounding systolic pressure in patients with chronic liver 316

- Ranges, Hilbert A. See CHASIS, REDISH, GOLDRING, RANGES, and SMITH 583
- Rapoport, S., and Guest, George M. The effect of salicylates on the electrolyte structure of the blood plasma. I. Respiratory alkalosis in monkeys and dogs after sodium and methyl salicylate; the influence of hypnotic drugs and of sodium bicarbonate on salicylate poisoning 759
— See GUEST, RAPOPORT, and ROSCOE 770
- Rawson, Rulon W., Moore, Francis D., Peacock, Wendell, Means, J. H., Cope, Oliver, and Riddell, Charlotte B. Effect of iodine on the thyroid gland in Graves' disease when given in conjunction with thiouracil—a two-action theory of iodine 869
- Redish, Jules. See CHASIS, REDISH, GOLDRING, RANGES, and SMITH 583
- Reifenstein, E. C., Jr., Forbes, A. P., Albright, F., Donaldson, E., and Carroll, E. Effect of methyl testosterone on urinary 17-ketosteroids of adrenal origin 416
- Reinhold, John G., Flippin, Harrison F., Zimmerman, Joseph J., Geftter, William I., and Riddler, John G. The relationship between concentration of sulfamerazine in body fluids and the response in treatment of meningococcic meningitis 352
- Rhode, C. Martin, Morales, Manuel F., and Lozner, Eugene L. Studies on the quantitative evaluation of certain treatments in the healing of experimental third degree burns 372
- Ricca, Renato A., Fink, K., Katzin, Leonard I., and Warren, Stafford L. Effect of environmental temperature on experimental traumatic shock in dogs 127
—, —, Steadman, L. T., and Warren, Stafford L. The distribution of body fluids of dogs in traumatic shock 140
—, —, and Warren, Stafford L. The effect of sulfadiazine, antitoxins, globulins, and dog plasma on dogs in traumatic shock under sodium pentobarbital anesthesia 146
— See KATZIN, RICCA, and WARREN 149
- Richards, D. W., Jr. See COUNNAND, RILEY, BREED, BALDWIN, and RICHARDS 106
- Riddell, Charlotte B. See RAWSON, MOORE, PEACOCK, MEANS, COPE, and RIDDELL 869
- Riddler, John G. See REINHOLD, FLIPPIN, ZIMMERMAN, GEFTTER, and RIDDLER 352
- Riegel, C. See FLETCHER, HARDY, RIEGEL, and KOOP 405
- Riggs, Douglas S., Man, Evelyn B., and Winkler, Alexander W. Serum iodine of euthyroid subjects treated with desiccated thyroid 722
— See WINKLER, RIGGS, and MAN 732
- Riley, R. L. See COUNNAND, RILEY, BREED, BALDWIN, and RICHARDS 106
- Robson, James S. See RALLI, ROBSON, CLARKE, and HOAGLAND 316
- Roscoe, C. See GUEST, RAPOPORT, and ROSCOE 770
- Rose, Harry M., Culbertson, James T., and Lipman, Miriam Olmstead. Antistreptolysin titers in cases of filariasis with recurrent lymphangitis among military personnel 532
- Rothbard, Sidney. See DOLE, WATSON, and ROTHBARD 648
- Rovenstine, E. A. See NEUMANN, FOSTER, and ROVENSTINE 345
- Rutstein, David D., Thomson, K. Jefferson, Tolmach, Daniel M., Walker, William H., and Floody, Robert J. Plasma volume and "extravascular thiocyanate space" in pneumococcus pneumonia 11
—, Stebbins, Robert B., Cathcart, Richard T., and Harvey, Rejane M. The absorption and excretion of streptomycin in human chronic typhoid carriers 898
- S
- Salk, Jonas E., Pearson, Harold E., Brown, Philip N., and Francis, Thomas, Jr. Protective effect of vaccination against induced influenza B 547
— See FRANCIS, SALK, PEARSON, and BROWN 536
- Samper, Bernardo A. See FINLAND, BARNES, and SAMPER 192
— See FINLAND, PETERSON, ALLEN, SAMPER, and BARNES 451
— See FINLAND, PETERSON, ALLEN, SAMPER, and BARNES 458
— See FINLAND, SAMPER, and BARNES 483
— See FINLAND, SAMPER, and BARNES 497
- Sandmeyer, John A. See QUINBY, DEXTER, SANDMEYER, and HAYNES 69
- Sappington, Thomas S. See GROSSMAN, SAPPINGTON, BURROWS, LAVIETES, and PETERS 523
- Scatchard, G., Strong, L. E., Hughes, W. L., Jr., Ashworth, J. N., and Sparrow, A. H. Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXVI. The properties of solutions of human serum albumin of low salt content 671
- Schleicher, E. M. See MOOSNICK, SCHLEICHER, and PETERSON 278
- Schmidt, Gerhard. See PROGER, DEKANEAS, and SCHMIDT 864
- Schoenbach, Emanuel B. See HARTLEY, ENDERS, MUELLER, and SCHOENBACH 92
- Scholz, Dale E., Schultz, John H., Pleune, F. Gordon, Fink, K., Steadman, L. T., and Warren, Stafford L. Study of the body temperature and water content in shock produced by the continuous intravenous injection of adrenalin, with and without anesthesia 154
- Schultz, John H. See SCHOLZ, SCHULTZ, PLEUNE, FINK, STEADMDN, and WARREN 154
- Schwartz, S. See PASS, SCHWARTZ, and WATSON 283

- Seligman, Arnold M. See FRANK, SELIGMAN, and FINE 435
- Smith, Homer W., Finkelstein, Norma, Aliminosa, Lucy, Crawford, Betty, and Graber, Martha. The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man 388
- See CHASIS, REDISH, GOLDRING, RANGES, and SMITH 583
- See BRADLEY, CHASIS, GOLDRING, and SMITH 749
- Snapp, E. F. See DRILL, ANNEGERS, SNAPP, and IVY 97
- Sparrow, A. H. See SCATCHARD, STRONG, HUGHES, ASHWORTH, and SPARROW 671
- Stare, F. J. See METCOFF, FAVOUR, and STARE 82
- See HEGSTED, HAY, and STARE 657
- Stats, Daniel. Cold hemagglutination and cold hemolysis. The hemolysis produced by shaking cold-agglutinated erythrocytes 33
- Stead, E. A., Jr., Warren, J. V., Merrill, A. J., and Brannon, E. S. The cardiac output in male subjects as measured by the technique of right atrial catheterization. Normal values with observations on the effect of anxiety and tilting 326
- See BRANNON, MERRILL, WARREN, and STEAD 332
- See WARREN, BRANNON, STEAD, and MERRILL 337
- Steadman, L. T. See SCHOLZ, SCHULTZ, PLEUNE, FINK, STEADMAN and WARREN 154
- See RICCA, FINK, STEADMAN, and WARREN 140
- Stebbins, Robert B. See RUTSTEIN, STEBBINS, CATHCART, and HARVEY 898
- Strong, L. E. See SCATCHARD, STRONG, HUGHES, ASHWORTH, and SPARROW 671
- Suksta, A. See CARTWRIGHT, WINTROBE, BUSCHKE, FOLLIS, SUKSTA, and HUMPHREYS 268
- Sweet, Lewis K. See DOWLING, MAYER, SWEET, and DUMOFF-STANLEY 160

T

- Tagnon, Henry J. The nature of the mechanism of the shock produced by the injection of trypsin and thrombin 1
- See TAYLOR, DAVIDSON, TAGNON, ADAMS, MACDONALD, and MINOT 698
- See MINOT, DAVIDSON, LEWIS, TAGNON, and TAYLOR 704
- Taylor, F. H. L., Davidson, C. S., Tagnon, H. J., Adams, M. A., MacDonald, A. H., and Minot, George R. Studies in blood coagulation: the coagulation properties of certain globulin fractions of normal human plasma *in vitro* 698
- See MINOT, DAVIDSON, LEWIS, TAGNON, and TAYLOR 704
- Taylor, Howard C., Jr. See KLOFF, YOUNG, and TAYLOR 117
- See KLOFF, YOUNG, and TAYLOR 150

- Thomas, C. B. See BING, THOMAS, and WAPLES 513
- Thomas, Lewis, Mirick, George S., Curnen, Edward C., Ziegler, James E., Jr., and Horsfall, Frank L., Jr. Studies on primary atypical pneumonia. II. Observations concerning the relationship of a non-hemolytic streptococcus to the disease 227
- See CURNEN, MIRICK, ZIEGLER, THOMAS, and HORSFALL 209
- Thomson, K. Jefferson. See RUTSTEIN, THOMSON, TOLMACH, WALKER, and FLOODY 11
- Thorn, G. W., Armstrong, S. H., Jr., Davenport, V. D., Woodruff, L. M., and Tyler, F. H. Chemical, clinical, and immunological studies on the products of human plasma fractionation. XXVII. The use of salt-poor concentrated human serum albumin solution in the treatment of chronic Bright's disease 802
- Tibbetts, D. M. See BRUES, COHN, KETY, NATHANSON, NUTT, TIBBETTS, ZAMECNIK, and AUB 838
- Tillett, William S., McCormack, James E., and Cambier, Margaret J. The treatment of lobar pneumonia with penicillin 589
- , —, and —. The use of penicillin in the local treatment of pneumococcal empyema 595
- Tolmach, Daniel M. See RUTSTEIN, THOMSON, TOLMACH, WALKER, and FLOODY 11
- Tyler, F. H. See THORN, ARMSTRONG, DAVENPORT, WOODRUFF, and TYLER 802

V

- van Herick, William. See MEIKLEJOHN, EATON, and VAN HERICK 241

W

- Walker, William H. See RUTSTEIN, THOMSON, TOLMACH, WALKER, and FLOODY 11
- Waples, E. C. See BING, THOMAS, and WAPLES 513
- Warren, J. V., Brannon, E. S., Stead, E. A., Jr., and Merrill, A. J. The effect of venerection and the pooling of blood in the extremities on the atrial pressure and cardiac output in normal subjects with observations on acute circulatory collapse in three instances 337
- See STEAD, WARREN, MERRILL, and BRANNON 326
- See BRANNON, MERRILL, WARREN, and STEAD 332
- Warren, Stafford L. See RICCA, FINE, KATZIN, and WARREN 127
- See RICCA, FINE, STEADMAN, and WARREN 140
- See RICCA, FINE, and WARREN 146
- See KATZIN, RICCA, and WARREN 142
- See KATZIN and WARREN 152
- See SCHOLZ, SCHULTZ, PLEUNE, FINK, STEADMAN, and WARREN 154
- Watson, C. J. See PARR, SCHWARTZ, and WATSON 243

- Watson, Robert F. See DOLE, WATSON, and ROTH-
BARD 648
- Webb, Joseph P. See ENGEL, WEBB, and FERRIS
687
- Wilkins, Lawson, and Fleischmann, Walter. Stud-
ies on the creatinuria due to methylated
steroids 21
- Winkler, Alexander W., Riggs, Douglas S., and
Man, Evelyn B. Serum iodine in hypothyroid-
ism before and during thyroid therapy 732
- See RIGGS, MAN, and WINKLER 722
- Wintrobe, M. M. See CARTWRIGHT, WINTROBE,
BUSCHKE, FOLLIS, SUKSTA, and HUMPHREYS
268
- Wolff, Harold G. See BIGELOW, HARRISON, GOOD-
ELL, and WOLFF 503
- Woodruff, L. M. See THORN, ARMSTRONG, DAVEN-
PORT, WOODRUFF, and TYLER 802

Y

- Young, Lawrence E., and Lawrence, John S.
Maturation and destruction of transfused
human reticulocytes. Evaluation of reticulo-
cyte experiments for the measurement of hemo-
globin metabolism 554
- Young, Nelson F. See KLOPP, YOUNG, and TAYLOR
117

- See KLOPP, YOUNG, and TAYLOR 189

Z

- Zamcheck, Norman. See FRANK, ALTSCHULE, and
ZAMCHECK 54
- Zamecnik, P. C., Aub, J. C., Brues, A. M., Kety,
S. S., Nathanson, I. T., Nutt, A. L., and Pope,
A. The toxic factors in experimental traumatic
shock. V. Chemical and enzymatic properties
of muscle exudate 853
- See NATHANSON, NUTT, POPE, ZAMECNIK, AUB,
BRUES, and KETY 829
- See BRUES, COHN, KETY, NATHANSON, NUTT,
TIBBETTS, ZAMECNIK, and AUB 838
- See KETY, NATHANSON, NUTT, POPE, ZAMECNIK,
AUB, and BRUES 842
- See AUB, BRUES, KETY, NATHANSON, NUTT,
POPE, and ZAMECNIK 848
- See POPE, ZAMECNIK, AUB, BRUES, DUBOS,
NATHANSON, and NUTT 859
- Ziegler, James E., Jr. See CURNEN, MIRICK,
ZIEGLER, THOMAS, and HORSFALL 209
- See THOMAS, MIRICK, CURNEN, ZIEGLER, and
HORSFALL 227
- Zimmerman, Joseph J. See REINHOLD, FLIPPIN,
ZIMMERMAN, GEFTER, and RIDDLER 352

SUBJECT INDEX

A

- Agglutinin response:** In meningococcic meningitis (DOWLING, MAYER, SWEET, and DUMOFF-STANLEY) 160
- Agglutinins:** Type-specific meningococcic (FALK and APPELBAUM) 742
- Albumin:** Human serum albumin of low salt content (SCATCHARD, STRONG, HUGHES, ASHWORTH, and SPARROW) 671
- , salt-poor, use of in Bright's disease (THORN, ARMSTRONG, DAVENPORT, WOODRUFF, and TYLER) 802
- , serum, as diluent for anti-Rh typing reagents (CAMERON and DIAMOND) 793
- Alkalosis:** Respiratory, after salicylates (RAPOPORT and GUEST) 759
- Amino acids:** Effect of on serum and urine creatine (GROSSMAN) 380
- Anemia:** Pyridoxine deficiency, from casein hydrolysate feeding (CARTWRIGHT, WINTROBE, BUSCHKE, FOLLIS, SUKSTA, and HUMPHREYS) 268
- , chronic, cardiac output in patients with (BRANNON, MERRILL, WARREN, and STEAD) 332
- , hemolytic, serological and physical features of cold agglutinins in (FINLAND, PETERSON, and BARNES) 474
- , of malaria, effect of alterations in blood volume on (FELDMAN and MURPHY) 780
- , refractory pernicious, treated with choline chloride (MOOSNICK, SCHLEICHER, and PETERSON) 278
- Anesthesia:** Spinal, compensating vasoconstriction in (NEUMANN, FOSTER, and ROVENSTINE) 345
- Anoxia:** Comparison with acute alcoholic intoxication and hypoglycemia (ENGEL, WEBB, and FERRIS) 687
- Anti-Rh serums:** Rh inhibitor substance in (DIAMOND and ABELSON) 122
- Antistreptolysin titers:** In filariasis with lymphangitis (ROSE, CULBERTSON, and LIPMAN) 532
- Ascites:** In patients with cirrhosis of the liver (RALLI, ROBSON, CLARKE, and HOAGLAND) 316
- Ascorbic acid:** With special reference to white layer (LUSCHKEZ) 573

B

- Basal metabolism:** Effect of histamine on (PETERS, HORTON, and BOOTHBY) 611
- Biliary fistula:** Effect of on liver function (DEILL, ANNEGEES, SNAPP, and IVY) 97
- Bilirubin:** Conversion of hematin to (PASS, SCHWARTZ, and WATSON) 253
- Blood coagulation:** Coagulation properties of human plasma fractions (TAYLOR, DAVIDSON, TAGNON, ADAMS, MACDONALD, and MINOT) 475

- Blood flow:** Hepatic, estimation of in man (BRADLEY, INGELFINGER, BRADLEY, and CURRY) 890
- Blood pressure:** Maintenance of by vasoconstriction during spinal anesthesia (NEUMANN, FOSTER, and ROVENSTINE) 345
- Blood volume:** Effect of alterations in on anemia and hypoproteinemia of malaria (FELDMAN and MURPHY) 780
- Body fluids:** Changes of in pneumococcus pneumonia (RUTSTEIN, THOMSON, TOLMACH, WALKER, and FLOODY) 11
- , in traumatic shock (RICCA, FINK, STEADMAN, and WARREN) 140
- Bright's disease:** Use of salt-poor albumin in treatment of (THORN, ARMSTRONG, DAVENPORT, WOODRUFF, and TYLER) 802
- Burns:** Third degree, quantification of healing of (RHODE, MORALES, and LOZNER) 372
- , nitrogen balance in experimental (MEYER, JOSEPH, HIRSHFELD, and ABBOTT) 579

C

- Calcium metabolism:** In nephrosis (EMERSON and BECKMAN) 564
- Cardiac output:** By right heart catheterization (COURNAND, RILEY, BREED, BALDWIN, and RICHARDS) 106
- , effect of venesection on (WARREN, BRANNON, STEAD, and MERRILL) 337
- , in normal subjects (STEAD, WARREN, MERRILL, and BRANNON) 326
- , in patients with chronic anemia (BRANNON, MERRILL, WARREN, and STEAD) 332
- Carriers:** Of streptococci, effect of tyrothricin on (HARTLEY, ENDERS, MUELLER, and SCHOENBACH) 92
- Casein hydrolysate:** And pyridoxine deficiency anemias (CARTWRIGHT, WINTROBE, BUSCHKE, FOLLIS, SUKSTA, and HUMPHREYS) 268
- Cephalin flocculation:** Positive, mechanism of in hepatitis (MOORE, PIERSON, HANGER, and MOORE) 292
- , reaction in malaria (GUTTMAN, POTTER, HANGER, MOORE, PIERSON, and MOORE) 295
- Choline chloride:** For treatment of refractory pernicious anemia (MOOSNICK, SCHLEICHER, and PETERSON) 278
- Circulation:** In experimental neurogenic hypertension (BING, THOMAS, and WARLEY) 513
- , and respiration during chill and fever (ALT-SCHULE, FRIEDBERG, and McMANUS) 578
- Cirrhosis:** Of the liver, ascites in patients with (RALLI, ROBSON, CLARKE, and HOAGLAND) 316
- Clothing:** Importance of solar heat load in design of (BLUM) 712

- Coagulation defect:** Effect in hemophilia of a plasma fraction (MINOT, DAVIDSON, LEWIS, TAGNON, and TAYLOR) 704
- Cold agglutinins:** In various conditions (FINLAND, PETERSON, ALLEN, SAMPER, and BARNES) 451
- —, deterioration of on storage (FINLAND and BARNES) 490
- —, for an indifferent streptococcus (FINLAND, SAMPER, and BARNES) 497
- —, method of determining (FINLAND, SAMPER, and BARNES) 483
- —, in primary atypical pneumonia (FINLAND, PETERSON, ALLEN, SAMPER, and BARNES) 458
- —, serological and physical features of (FINLAND, PETERSON, and BARNES) 474
- Cold hemagglutination:** And cold hemolysis (STATS) 33
- Cold hemolysis:** Cold hemagglutination and (STATS) 33
- Complement fixation:** In human malaria (LIPPINCOTT, GORDON, HESSELBROCK, and MARBLE) 362
- Constriction:** Of human renal artery, effect of (QUINBY, DEXTER, SANDMEYER, and HAYNES) 69
- Creatine:** Serum and urine, effect of amino acids on (GROSSMAN) 380
- Creatinuria:** Due to methylated steroids (WILKINS and FLEISCHMANN) 21
- Cytochrome C:** Injected, observations on effect of in animals (PROGER, DEKANEAS, and SCHMIDT) 864

D

- Decholin:** Versus mercupurin as diuretics (MODELL and GOLD) 384
- Diuretics:** Decholin versus mercupurin (MODELL and GOLD) 384

E

- Electrocardiograms:** In traumatic shock (BURNETT, BLAND, and BEECHER) 694
- Electroencephalographic studies:** Quantitative, of anoxia (ENGEL, WEBB, and FERRIS) 687
- Electrolyte balance:** In shock (BRUES, COHN, KETY, NATHANSON, NUTT, TIBBETTS, ZAMECNIK, and AUB) 838
- Electrolyte structure:** Of plasma, effect of salicylates on (GUEST, RAPOPORT, and ROSCOE) 770
- Electrophoretic analysis:** Of maternal and fetal plasmas and sera (LONGSWORTH, CURTIS, and PEMBROKE) 46
- Electrophoretic changes:** In plasma protein patterns in malaria (DOLE and EMERSON) 644
- —, in serum protein patterns in scarlet fever and rheumatic fever (DOLE, WATSON, and ROTHBARD) 648
- Empyema:** Pneumococcal, treated with penicillin (TILLET, McCORMACK, and CAMBIER) 595

- Endocarditis:** Subacute, bacterial, treated with penicillin (BLOOMFIELD, ARMSTRONG, and KIRBY) 251

F

- Fibrinogen:** Increasing factor obtained from sterile pus (HOMBURGER) 43
- Filariosis:** Antistreptolysin titers in with lymphangitis (ROSE, CULBERTSON, and LIPMAN) 532

G

- Gelatin:** As a plasma substitute (FLETCHER, HARDY, RIEGEL, and KOOP) 405
- Globulin fractions:** Coagulation properties of *in vitro* (TAYLOR, DAVIDSON, TAGNON, ADAMS, MACDONALD, and MINOT) 698
- Graves' disease:** Effect of iodine, with thiouracil, on thyroid in (RAWSON, MOORE, PEACOCK, MEANS, COPE, and RIDDELL) 869

H

- Heart:** Right, catheterization of to measure cardiac output (COURNAND, RILEY, BREED, BALDWIN, and RICHARDS) 106
- , Increased plasma volume in cardiac insufficiency (PERERA) 708
- Hematin:** Conversion of to bilirubin (PASS, SCHWARTZ, and WATSON) 283
- Hemodynamic alterations:** During the pyrogenic reaction (BRADLEY, CHASIS, GOLDRING, and SMITH) 749
- Hemoglobin:** And plasma protein in protein-deficient rat (METCOFF, FAVOUR, and STARE) 82
- Hemophilia:** Effect of a fraction of plasma globulins (MINOT, DAVIDSON, LEWIS, TAGNON, and TAYLOR) 704
- Hepatic diseases:** Lipids of serum and liver in (MAN, KARTIN, DURLACHER, and PETERS) 623
- Hepatitis:** Mechanism of positive cephalin flocculation in (MOORE, PIERSON, HANGER, and MOORE) 292
- Hippuric acid derivatives:** Clearances of (SMITH, FINKELSTEIN, ALIMINOSA, CRAWFORD, and GRABER) 388
- Histamine:** Effect of on basal metabolism (PETERS, HORTON, and BOOTHBY) 611
- Hypertensin:** Preparation and assay of (DEXTER, HAYNES, and BRIDGES) 62
- Hypertensinase:** Content of human plasma (HAYNES and DEXTER) 75
- Hypertensinogen:** Preparation and assay of (DEXTER, HAYNES, and BRIDGES) 62
- , content of human plasma (HAYNES and DEXTER) 78
- Hypertension:** Circulation in experimental neurogenic (BING, THOMAS, and WAPLES) 513
- Hypoparathyroidism:** Treatment of in dogs (McCHESNEY and GIACOMINO) 680

- Hypoproteinemia:** In swine fed casein hydrolysate
(CARTWRIGHT, WINTROBE, BUSCHKE, FOLLIS,
SUKSTA, and HUMPHREYS) 268
—, of malaria, effect of alterations in blood volume
on (FELDMAN and MURPHY) 780
Hypothyroidism: Serum iodine in (WINKLER, RIGGS,
and MAN) 732

I

- Influenza:** Virus isolations and serological studies
(FINLAND, BARNES, and SAMPER) 192
Influenza A: Vaccination against (FRANCIS, SALK,
PEARSON, and BROWN) 536
Influenza B: Vaccination against (SALK, PEARSON,
BROWN, and FRANCIS) 547
Iodine: Serum, of euthyroid subjects on desiccated
thyroid (RIGGS, MAN, and WINKLER) 722
—, effect of, with thiouracil, on thyroid in Graves'
disease (RAWSON, MOORE, PEACOCK, MEANS,
COPE, and RIDDELL) 869
—, serum, in hypothyroidism (WINKLER, RIGGS,
and MAN) 732
Isoagglutinin: Anti-A reagents from mixed blood of
groups O and B (MELIN) 662

K

- 17-Ketosteroids:** Effect of methyl testosterone on
(REIFENSTEIN, FORBES, ALBRIGHT, DONALD-
SON, and CARROLL) 416
Kidney: Assay of renin, hypertensinogen, and hyper-
tensin (DEXTER, HAYNES, and BRIDGES) 62
—, effect of constriction of human renal artery
(QUINBY, DEXTER, SANDMEYER, and HAYNES) 69
—, hypertensinase content of human plasma
(HAYNES and DEXTER) 75
—, renal clearances of hippuric acid derivatives
(SMITH, FINKELSTEIN, ALIMINOSA, CRAWFORD,
and GRABER) 388
Kidney function: Errors in measurement of (KLOPP,
YOUNG, and TAYLOR) 117
— —, evaluated by means of sodium p-amino-
hippurate (CHASIS, REDISH, GOLDRING, RANGES,
and SMITH) 583
— —, and testosterone (KLOPP, YOUNG, and
TAYLOR) 189

L

- Lipids:** Of serum and liver in hepatic diseases (MAN,
KARTIN, DURLACHER, and PETERS) 623
Liver: Arcites in patients with cirrhosis of (RALLI,
ROBSON, CLARKE, and HOAGLAND) 316
—, function tests in malaria (LIPPINCOTT, ELLER-
BROOK, HESSELBROCK, GORDON, GOTTLIEB, and
MARBLE) 616
Liver function: Effect of biliary fistula on (DEILL,
ANNIGERS, SNAPP, and IVY) 67
Lymphangitis: Anti-treptolysin titers in patients
with (ROSE, CULBERTSON, and LILHAN) 532

M

- Malaria:** Cephalin flocculation reaction in (GUTT-
MAN, POTTER, HANGER, MOORE, PIERSON, and
MOORE) 296
—, changes in plasma protein patterns in (DOLE
and EMERSON) 644
—, complement fixation in (LIPPINCOTT, GORDON,
HESSELBROCK, and MARBLE) 362
—, effect of alterations in blood volume on ane-
mia and hypoproteinemia of (FELDMAN and
MURPHY) 780
—, liver function tests in (LIPPINCOTT, ELLER-
BROOK, HESSELBROCK, GORDON, GOTTLIEB, and
MARBLE) 616
Meningitis: Meningococcus, agglutinin response in
(DOWLING, MAYER, SWEET, and DUMOFF-
STANLEY) 160
—, sulfamerazine level and response in (REINHOLD,
FLIPPIN, ZIMMERMAN, GEFTER, and RIDDLE) 352
Mercupurin: Versus decholin as diuretics (MODELL
and GOLD) 384
Muscle exudate: Chemical and enzymatic properties
of (ZAMECNIK, AUB, BRUES, KETY, NATHANSON,
NUTT, and POPE) 853
Muscle ischemia: And loss of vascular fluid, with
shock (KETY, NATHANSON, NUTT, POPE, ZAMEC-
NIK, AUB, and BRUES) 842
Muscle, ischemic: Effects of intravenous injection of
effusion from (AUB, BRUES, KETY, NATHANSON,
NUTT, POPE, and ZAMECNIK) 848
—, —, bacterial flora of exudates from and their
toxicity (POPE, ZAMECNIK, AUB, BRUES, DUROS,
NATHANSON, and NUTT) 859
Muscle ligation: Physiologic effects of (NATHANSON,
NUTT, POPE, ZAMECNIK, AUB, BRUES, and
KETY) 829

N

- Nephrosis:** Calcium metabolism in (EMERSON and
BECKMAN) 564
Nitrogen: Metabolism in acute infections (GROSS-
MAN, SAPPINGTON, BURROWS, LAVIETES, and
PETERS) 523
Nitrogen balance: In experimental burns (MEYER,
JOSEPH, HIRSHFELD, and ARON) 579
Nutritive value: Of human plasma fractions (HIG-
STED, HAY, and STAKE) 657

- , in local treatment of pneumococcal empyema (TILLET, McCORMACK, and CAMBIER) 595
- , treatment of lobar pneumonia with (TILLET, McCORMACK, and CAMBIER) 589
- , treatment of subacute bacterial endocarditis with (BLOOMFIELD, ARMSTRONG, and KIRBY) 251
- Plasma:** Hypertensinase content of (HAYNES and DEXTER) 75
- , anti-A isoagglutinin reagents from mixed blood of groups O and B (MELIN) 662
- , effect of salicylates on electrolyte structure of (GUEST, RAPOPORT, and ROSCOE) 770
- , fractionation, albumin as diluent for anti-Rh typing reagents (CAMERON and DIAMOND) 793
- , fractionation, use of salt-poor albumin in Bright's disease (THORN, ARMSTRONG, DAVENPORT, WOODRUFF, and TYLER) 802
- , gelatin as a plasma substitute (FLETCHER, HARDY, RIEGEL, and KOOP) 405
- , hypertensinogen content of (HAYNES and DEXTER) 78
- , nutritive value of fractions (HEGSTED, HAY, and STARE) 657
- Plasma, fetal:** Electrophoretic analysis of (LONGSWORTH, CURTIS, and PEMBROKE) 46
- Plasma globulins:** Effect of a fraction of in hemophilia (MINOT, DAVIDSON, LEWIS, TAGNON, and TAYLOR) 704
- Plasma, maternal:** Electrophoretic analysis of (LONGSWORTH, CURTIS, and PEMBROKE) 46
- Plasma protein:** And hemoglobin in protein-deficient rat (METCOFF, FAVOUR, and STARE) 82
- Plasma protein patterns:** In malaria (DOLE and EMERSON) 644
- Plasma volume:** Increased in cardiac insufficiency (PERERA) 708
- Pneumonia:** Pneumococcus, plasmavolume and "extravascular thiocyanate space" in (RUTSTEIN, THOMSON, TOLMACH, WALKER, and FLOODY) 11
- , atypical, clinical and laboratory studies of (CURNEN, MIRICK, ZIEGLER, THOMAS, and HORSFALL) 209
- , atypical, cold agglutinins in (FINLAND, PETERSON, ALLEN, SAMPER, and BARNES) 458
- , atypical, epidemic of acute respiratory disease with (BRESLOW) 775
- , atypical, experimental transmission of (COMMISSION ON ACUTE RESPIRATORY DISEASES) 175
- , atypical, due to a new virus (MEIKLEJOHN, EATON, and VAN HERICK) 241
- , atypical, serological and physical features of cold agglutinins in (FINLAND, PETERSON, and BARNES) 474
- , atypical, and streptococcus MG (THOMAS, MIRICK, CURNEN, ZIEGLER, and HORSFALL) 227
- , lobar, treatment with penicillin of (TILLET, McCORMACK, and CAMBIER) 589

R

- Renin:** Preparation and assay of (DEXTER, HAYNES, and BRIDGES) 62
- Respiration:** And circulation during chill and fever (ALTSCHULE, FREEDBERG, and McMANUS) 878
- Respiratory disease:** Epidemic of with atypical pneumonia (BRESLOW) 775
- Reticulocytes:** Maturation and destruction of (YOUNG and LAWRENCE) 554
- Rh, anti-:** Typing reagents, albumin as diluent for (CAMERON and DIAMOND) 793
- Rh inhibitor:** Substance in anti-Rh serums (DIAMOND and ABELSON) 122
- Rheumatic fever:** Electrophoretic changes in serum protein patterns in (DOLE, WATSON, and ROTHBARD) 648

S

- Salicylates:** Respiratory alkalosis after sodium and methyl salicylate (RAPOPORT and GUEST) 759
- , effect of on the electrolyte structure of plasma (GUEST, RAPOPORT, and ROSCOE) 770
- Scarlet fever:** Electrophoretic changes in serum protein patterns in (DOLE, WATSON, and ROTHBARD) 648
- Serum:** Iodine of euthyroid subjects treated with desiccated thyroid (RIGGS, MAN, and WINKLER) 722
- , iodine in hypothyroidism (WINKLER, RIGGS, and MAN) 732
- Serum protein patterns:** Electrophoretic changes in in scarlet fever and rheumatic fever (DOLE, WATSON, and ROTHBARD) 648
- Shock:** Produced by injection of trypsin and thrombin (TAGNON) 1
- , accompanying muscle ischemia and loss of vascular fluid (KETTY, NATHANSON, NUTT, POPE, ZAMECNIK, AUB, and BRUES) 842
- , adrenalin, body temperature and water content in (SCHOLZ, SCHULTZ, PLEUNE, FINK, STEADMAN, and WARREN) 154
- , bacterial flora of ischemic muscle exudates and their toxicity (POPE, ZAMECNIK, AUB, BRUES, DUBOS, NATHANSON, and NUTT) 859
- , chemical and enzymatic properties of muscle exudate (ZAMECNIK, AUB, BRUES, KETY, NATHANSON, NUTT, and POPE) 853
- , effects of intravenous injection of effusion from ischemic muscle (AUB, BRUES, KETY, NATHANSON, NUTT, POPE, and ZAMECNIK) 848
- , electrolyte and water balance in (BRUES, COHN, KETY, NATHANSON, NUTT, TIBBETTS, ZAMECNIK, and AUB) 838
- , hemorrhagic, intestinal absorption in (GOLDBERG and FINE) 445
- , physiologic effects of muscle ligation (NATHANSON, NUTT, POPE, ZAMECNIK, AUB, BRUES, and KETY) 829

- , therapy of irreversible hemorrhagic (FRANK, SELIGMAN, and FINE) 435
 - , tourniquet, environmental temperature in (KATZIN, RICCA, and WARREN) 149
 - , tourniquet, thiamine in (KATZIN and WARREN) 152
 - , traumatic, body fluids in (RICCA, FINK, STEADMAN, and WARREN) 140
 - , traumatic, electrocardiograms in (BURNETT, BLAND, and BEECHER) 694
 - , traumatic, environmental temperature in (RICCA, FINK, KATZIN, and WARREN) 127
 - , traumatic, paredrine therapy in (FRANK, ALTSCHULE, and ZAMCHECK) 54
 - , traumatic, sulfadiazine, etc., in (RICCA, FINK, and WARREN) 146
 - Sodium p-aminohippurate: Renal evaluation by means of (CHASIS, REDISH, GOLDRING, RANGES, and SMITH) 583
 - Solar heat load: Relationship of to total heat load and design of clothing (BLUM) 712
 - Staphylococci: Anti-biotic actions of penicillin on (KIRBY) 165
 - , penicillin inactivator from (KIRBY) 170
 - Steroids, methylated: Creatinuria due to (WILKINS and FLEISCHMANN) 21
 - Streptococcus: Hemolytic, effect of tyrothricin on carriers of (HARTLEY, ENDERS, MUELLER, and SCHOENBACH) 92
 - , an indifferent, agglutinins for (FINLAND, SAMPER, and BARNES) 497
 - Streptococcus MG: And atypical pneumonia (THOMAS, MIRICK, CURNEN, ZIEGLER, and HORSFALL) 227
 - Streptomycin: Absorption and excretion of in typhoid carriers (RUTSTEIN, STEBBINS, CATHCART, and HARVEY) 898
 - Sulfadiazine: In traumatic shock (RICCA, FINK, and WARREN) 146
 - Sulfamerazine: Level and response in meningitis (REINHOLD, FLIPPIN, ZIMMERMAN, GEFTER, and RIDDLE) 352
 - Sulfonamides: Chemical changes of in man (GILLIGAN) 301
- T
- Temperature: Environmental, in traumatic shock (RICCA, FINK, KATZIN, and WARREN) 127
 - , environmental, in tourniquet shock (KATZIN, RICCA, and WARREN) 149
- Testosterone: And renal functions in man (KLOPP, YOUNG, and TAYLOR) 189
 - , methyl, effect of on 17-ketosteroids (REIFENSTEIN, FORBES, ALBRIGHT, DONALDSON, and CARROLL) 416
 - Thiamine: In tourniquet shock (KATZIN and WARREN) 152
 - Thiouracil: Given with iodine in Graves' disease (RAWSON, MOORE, PEACOCK, MEANS, COPE, and RIDDELL) 869
 - Thrombin: Nature of shock produced by injection of (TAGNON) 1
 - Thyroid: Serum iodine of euthyroid subjects on desiccated thyroid (RIGGS, MAN, and WINKLER) 722
 - , in Graves' disease, effect of iodine with thiouracil on (RAWSON, MOORE, PEACOCK, MEANS, COPE, and RIDDELL) 869
 - , serum iodine in hypothyroidism (WINKLER, RIGGS, and MAN) 732
 - Trypsin: Nature of shock produced by injection of (TAGNON) 1
 - Typhoid carriers: Absorption and excretion of streptomycin in (RUTSTEIN, STEBBINS, CATHCART, and HARVEY) 898
 - Tyrothricin: Effect of on carriers of streptococci (HARTLEY, ENDERS, MUELLER, and SCHOENBACH) 92
- V
- Vaccination: Against influenza A (FRANCIS, SALK, PEARSON, and BROWN) 536
 - , against influenza B (SALK, PEARSON, BROWN, and FRANCIS) 547
 - Vascular fluid: loss of, and muscle ischemia, with shock (KETY, NATHANSON, NUTT, POPE, ZAMECNIK, AUB, and BRUES) 842
 - Vasoconstriction: Compensating, in spinal anesthesia (NEUMANN, FOSTER, and ROVENSTINE) 345
 - Venesection: Effect of on cardiac output (WARREN, BRANNON, STEAD, and MERRILL) 337
- W
- Water balance: In shock (BRUES, COHN, KETY, NATHANSON, NUTT, TIBBETTS, ZAMECNIK, and AUB) 838
- Z
- Zein: And pyridoxine deficiency anemias (CARTWRIGHT, WINTROBE, BUSCHKE, FOLLIS, SWEETA, and HUMPHREYS) 268

THIAMINE-DEFICIENT DIET IN TOURNIQUET SHOCK IN RATS¹

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INTRODUCTION

It has been reported (1) that body thiamine levels play a very important rôle in the resistance to hemorrhage shock in dogs. This report describes an attempt to confirm the rôle of thiamine in the case of tourniquet shock in rats. The tourniquet procedure has been well standardized (2). Using young adult rats under such a standardized procedure, it is possible to obtain a statistically valid analysis of the rôle of thiamine in tourniquet shock. While a comparison may or may not be made between two types of shock in two different species, we feel that these negative results are at least typical of tourniquet shock in rats.

METHODS

"Shock" was produced by placing a rubber-band tourniquet on one hind limb of a rat, leaving it for a stated period of time, and then removing the tourniquet. Upon removal, the animal goes into "shock," which under properly chosen conditions is fatal. The proportion of fatalities obtained is determined by the amount of tissue included under the tourniquet, the length of time the tourniquet is in position, and the environmental temperature, among other things. In these experiments, the tourniquet was applied to one hind limb only, usually anchoring it over the head of the femur. This served both to standardize the location and to prevent the band from slipping down to a lower position. After preliminary experiments, a length of time during which the tourniquet was in position was chosen such that only a small proportion of the normal rats used would suffer a fatal outcome to shock. Under these circumstances, if the thiamine-depleted animals proved more susceptible to shock, they would show a definitely higher death rate.

Litters of young rats were divided into control and test groups. The controls were fed the standard laboratory diet containing adequate thiamine, the others were fed

the thiamine-deficient diet used by earlier workers (1): 18.6 per cent vitamin-free casein, 58.8 per cent sucrose, 7.8 per cent cottonseed oil, 3.9 per cent salt mixture, 7.8 per cent autoclaved yeast, 1.0 per cent agar, and 2.0 per cent cod-liver oil. Experiments were performed after various periods on this diet.

No tissue analyses for thiamine were made, nor was the autoclaved yeast analyzed. However, many observations clearly indicated dietary effects on the rats fed the synthetic diet. These animals lost their appetites to a large extent, lost weight, became irritable and timorous. Their coats became matted and dirty-looking. One had no difficulty telling normally fed rats from those on the synthetic diet. In addition to feeding the synthetic diet, coprophagia was minimized by using cages with mesh floors, through which the fecal pellets dropped. An occasional surviving animal was offered the standard "dog chow" diet which it proceeded to attack ravenously.

No attempt was made to regulate the environmental temperature or other environmental factors, all controls being run simultaneously under the same laboratory conditions as the corresponding test group.

RESULTS

The results obtained are summarized in Table I. Of 24 animals, all survived a tourniquet in place for 4 to 4¼ hours. Of 88 animals sub-

TABLE I
Effect of thiamine-deficient diet on tourniquet shock survival in young adult rats

Tourniquet duration	Controls		B ₁ -deficients		
	Number of rats	Number succumbed	Days on diet	Number of rats	Number succumbed
4 hours	8 ♀	0	7	8 ♀	0
	4 ♀	0	16	4 ♂	0
4¼ hours	12 ♀	1	11	12 ♀	3
	7 ♀	3	14	7 ♀	3
	6 ♂	4	16	4 ♀ 4 ♂	3
	11 ♀	2	22	10 ♀	3
	7 ♀	6	39	8 ♀	4
	Totals	55	16	57	16

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Rochester School of Medicine and Dentistry. Part of the work was also assisted by grants in aid from the Rockefeller Foundation and the Fluid Research Fund of the University of Rochester.

jected to tourniquet for $4\frac{3}{4}$ hours, one third succumbed to "shock." It made no difference in this series whether the diet had been the normal laboratory diet or whether it was the thiamine-deficient synthetic diet. All animals that died succumbed in less than 36 hours following removal of the tourniquet.

Inasmuch as the transition between no fatalities and approximately 100 per cent fatality occurs in the short interval between approximately $4\frac{1}{4}$ hours tourniquet time, and approximately 5 to $5\frac{1}{2}$ hours tourniquet time, and a variation of resistance occurs between ages and between litters, a certain amount of variation in percentage fatality in different experiments with small numbers of animals may be expected. Individual experiments, however, always have been comparisons of sets of litter mates, and show no significant differences in death rates between normally fed animals and those on the deficient diet.

From this series of experiments, it may be concluded that thiamine plays at best a minor rôle in determining resistance to tourniquet shock in rats. One respect in which the thiamine-deficient animals may differ from the normals is

in their apparently greater tendency to gastric hemorrhage. Those animals that survive 15 hours or longer seem to show progressively greater gastric hemorrhage. The intestine in almost all cases also contains dark material suggesting partially digested blood. Frequently, the feces of animals going into shock give a positive blood reaction to benzidine reagent. This reaction seems more frequent with the thiamine-deficient animals than with the controls, although it is occasionally also found in the latter following shock. The feces fail to show the reaction before the shock treatment.

SUMMARY

In rats, a thiamine-deficient diet for various periods (11 to 39 days) had no appreciable influence on the survival rate from shock produced by a tourniquet.

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STUDY OF THE BODY TEMPERATURE AND WATER CONTENT IN SHOCK PRODUCED BY THE CONTINUOUS INTRAVENOUS INJECTION OF ADRENALIN, WITH AND WITHOUT ANESTHESIA¹

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INTRODUCTION

It has been pointed out (1) that all factors which are at present known to produce shock or to aggravate the condition if present have one physiological action in common. They are all adequate stimuli for producing hyperactivity of the sympathetic nervous system. More recently, a report (2) has been made that prolonged adrenalin injection produces a rather standardized reproducible shock-like condition, with a depressed blood pressure, increased hematocrit, and reduced blood volume. The observations of these workers have been repeated, using this method to obtain a reproducible state of shock where it was possible to record tissue temperatures and the percentage of total water in various tissues.

METHODS

Dogs weighing from 9 to 28 kgm. were used in this study. They were not limited as to breed, sex, or age, but, in general, young dogs in good health were selected. On the days of the experiment, the animals were not fed, but were allowed to drink water.

Nembutal was used in the experiments performed under anesthesia. A dose of 30 mgm. per kgm. was administered intraperitoneally at the beginning of the experiment, and, in some cases, an additional dose was given at subsequent intervals to maintain the anesthesia. In those experiments without general anesthesia, the dogs were strapped on the table and both the femoral arteries and veins were ex-

posed under a local anesthesia of 2 per cent procain. The right femoral artery was cannulated and connected to a mercury manometer for continuous blood pressure measurement in both groups of animals.

Constant temperature records of various muscles were made at frequent intervals during the whole experiment with specially constructed needle thermocouple equipment. After the control blood samples were taken and the control blood pressures and temperatures for various sites were recorded for 1 hour, the injection of adrenalin was started into the left femoral vein. A Murphy drip apparatus was regulated to deliver the adrenalin at a uniform rate. A 1:5000 dilution of adrenalin was used, and the usual rate was 0.010 mgm. per kgm. per minute for one hour. At the end of the hour, 10 ml. of saline were run through the apparatus into the vein to wash in the last 4 or 5 ml. of adrenalin solution remaining in the rubber tube leading to the needle.

After the dogs expired or were sacrificed, an autopsy was performed at which time duplicate samples of various tissues were taken for the determination of the percentage of total water. This was calculated from the weights of the tissue samples before and after drying at 100° C. for 48 hours.

RESULTS AND DISCUSSION

In studying temperature changes associated with shock, it is necessary to know the effect of the anesthesia used. This was studied by first recording the temperature changes occurring in anesthetized dogs. In order to know precisely the temperature changes associated with the adrenalin shock state, one group of animals was given adrenalin with no anesthesia.

One unanesthetized dog was studied over a period of 15 hours. The thermocouples were inserted and the temperatures recorded every 15 minutes. The rectal temperature remained constant within $\pm 0.2^{\circ}$ C. for most of the 15-hour period. The subcutaneous and intramuscular (both trunk and limb) temperatures paralleled each other very closely, there being no appreci-

¹ This study was assisted in part by a grant from the Josiah Macy Foundation. Part of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Rochester School of Medicine and Dentistry. Part of the work was also assisted by grants in aid from the Rockefeller Foundation and the Fluid Research Fund of the University of Rochester.

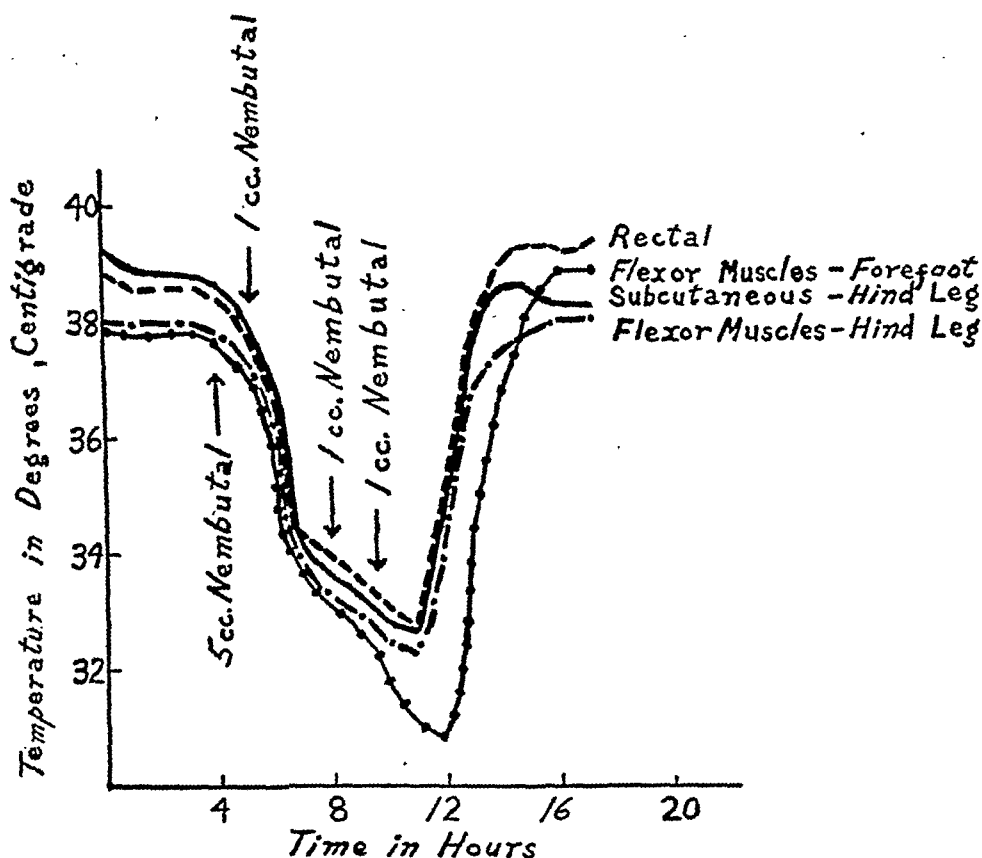


FIG. 1. TEMPERATURE CHANGES AS A RESULT OF NEMBUTAL ANESTHESIA IN A DOG
The Nembutal was injected intraperitoneally over a control period of 4 hours.

able difference between the two. Generally, they were from 0.5°C. to 1.0°C. lower than the rectal temperature.

Three dogs were given Nembutal intraperitoneally after a control period of 4 hours. A typical experiment is shown in Figure 1, which shows the effect of Nembutal to be a generalized parallel fall in the subcutaneous, intramuscular, and rectal temperatures.

Figure 2 represents the average temperature changes in 6 dogs which received adrenalin but no anesthesia. The rectal temperature remained at a constant of 39.9°C. The spinal muscles of the lumbar region followed closely the rectal temperature at 39.5°C. , with a tendency to fall slightly after the administration of adrenalin. The flexor muscles of the left hind leg fell steadily in temperature at the rate of 0.3°C. per hour, and the extensor muscles of the foreleg dropped in a parallel fashion. The temperature of the small muscle groups of the extremities

(flexor muscles of the forefoot and the extensor muscles of the hind foot) dropped over 1.5°C. during the period of an hour when adrenalin was injected. After the injection of adrenalin was completed, these two groups of muscles lost heat at the rate of about 0.2°C. per hour.

Figure 3 illustrates the behavior of the rectal and intramuscular temperatures in adrenalin shock with Nembutal anesthesia. The curves represent an average of 5 experiments. There are several points of difference in this group of animals compared with the group which received adrenalin without anesthesia. The level of the initial temperatures was roughly 2°C. lower. The rectal temperature fell during and after the adrenalin injection. The temperature of the extensor muscles of the foreleg, the flexor muscles of the hind leg, and the extensor muscles of the hind foot rose during the second hour after the completion of the adrenalin injection.

Thirty-two animals receiving adrenalin injec-

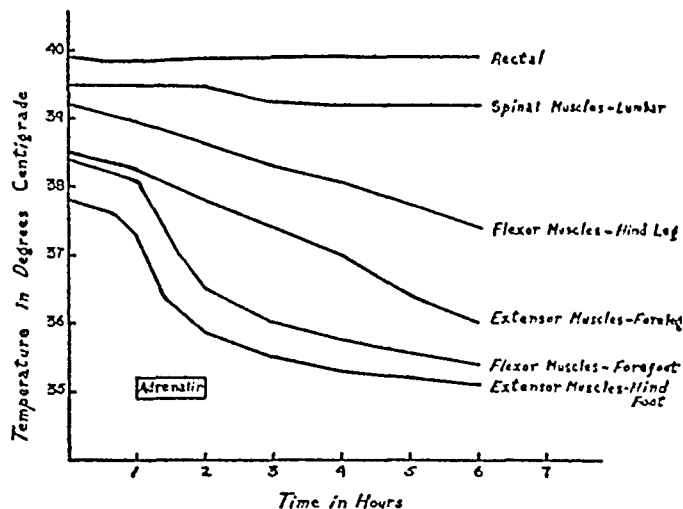


FIG. 2. TEMPERATURE CHANGES AS A RESULT OF ADRENALIN

The curves represent average values for 6 dogs. No anesthesia was given. Adrenalin was given at a uniform rate (0.010 mgm. per kgm. per minute) over a period of 1 hour following a control period of 1 hour.

tions have been studied. The results are summarized in Table I. On the basis of survival time, it appears that Nembutal anesthesia has a deleterious effect in adrenalin shock. If the amount of adrenalin was increased, the survival time was lessened. An attempt was made to raise the peripheral temperatures to normal by application of heat by means of lamps to the 4 extremities. The length of survival of the 5 animals studied shows that the heat applied to the extremities has no beneficial effect in adrenalin shock. Only 3 dogs were studied in which cold applications were made, and the results are inconclusive.

The hematocrit changes are also given in Table I. In 3 animals there was no significant change. The remaining dogs, however, showed an increase in hematocrit ranging from 5 to 30 per cent. The hematocrit did not show any uniform relationship to the degree of shock or survival time of the animal. It seemed to depend on characteristic individual differences in the vascular system and their responses to the experimental procedure. The wide variation in response to adrenalin and other drugs is well known clinically as well as experimentally.

The increase in hematocrit as a result of the adrenalin injections could be due to a loss of plasma into the tissues, to a release of red cells

from the spleen and splanchnic area, or to a withdrawal of plasma into the surface films in small vessels by vasoconstriction.

In some animals, the hematocrit may rise as much as 20 per cent within a few minutes after the adrenalin injection is started. It hardly seems reasonable that a loss of plasma into the tissues would occur so rapidly to account for this increased hematocrit. It has been shown in normal men (3) and in unanesthetized dogs (4) that there are no considerable stores of immobilized erythrocytes. The latter group concluded that all the red blood cells of the dog are in active circulation. It should be pointed out, however, that these conclusions may not apply to anesthetized dogs. The most logical explanation of the increase in hematocrit is a removal of plasma into the surface films of the small vessels by vasoconstriction. These workers have emphasized the importance of considering the sluggishly flowing plasma films in studies of plasma volume. They have also shown that the average hematocrit of the entire vascular system is considerably lower than the hematocrit of the large vessels, and the hematocrit of the smaller vessels is still less.

The arterial pressures are given in Table I. Only the normal levels and those at 30 minutes and 1 hour after completion of the adrenalin

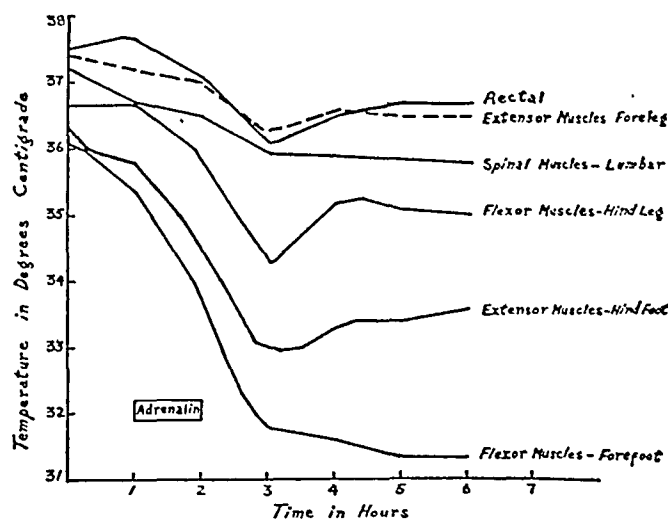


FIG. 3. TEMPERATURE CHANGES IN ADRENALIN SHOCK WITH NEMBUTAL ANESTHESIA

The curves represent average values for 6 dogs. Nembutal was given intraperitoneally. After 1 hour, the adrenalin was injected at a uniform rate (0.010 mgm. per kgm. per minute) over a period of 1 hour.

TABLE I

A summary of the results of adrenalin shock in 32 animals

Adrenalin dose in	Survival time after adrenalin injection was begun	Hematocrit			Blood pressure			Change in serum electrolytes		
		Normal	Immediately after completion of adrenalin injection	Pre-exitus	Normal	Hours after completion of adrenalin injection		K	Mg	Na
						½	1			
mgm. per kgm. per min. for 60 min.	hours min.	per cent			mm. Hg			per cent		

Seven dogs with Nembutal and standard dose of adrenalin

0.010	8	32	40	52	54	130	50	40	-3	+21	-5
0.010	4	43	47	63	62	150	100	85	-2	+28	+4
0.010	2	7	57	58	59	170	110	40	+1	+1	+2
0.010	1	20	52	70	68				+5	+31	+9
0.010	6	30	56	60	64	140	90	80			
0.010	1	25	52	50	50	115	Died				
0.010	0	48	52	Died		90	Died				

Ten dogs with no anesthesia and standard dose of adrenalin

0.009	Indefinitely	49	51		150	50	60			
0.010	Indefinitely	57	62		150	100	80			
0.010	1 56	40	47	47	150	50	Died	-2	-2	0
0.008	5 30	40	40	39	165	70	90	+7	-25	-4
0.010	Indefinitely	48	60		130	50	40			
0.010	Indefinitely	42	60		130	80	70	-3	+7	0
0.010	Sacrificed	52	56	57	170	40	35	-4	-5	-1
0.010	Sacrificed	44	44	44	90	35	35	+2	+2	+2
0.010	Sacrificed	50	53	56	170	100	95			
0.010	2 28	62	69	69	180	55	35	+15	+25	-2

Seven dogs with Nembutal and larger dose of adrenalin

0.011(112'')	1	30	45	53	53	140	50	Died	+7		
0.020 (40'')	0	40	49	63	63						
0.025	6	Sacrif.	42	56	56	150	70	60			
0.028 (95'')	2	45	38	46	45	145	80	40			
0.023(114'')	3	3	32	52	49	140	30	20			
0.029	1	0	44	50	50	130	Died		+5	+23	+3
0.011 (90'')	1	33	44	53	53	150	Died				

Five dogs with heat applied after adrenalin injection

0.010	2	5	53	63	63	140	70	55			
0.010	2	15	46	53	53	120	55	70			
0.011(45'')*	1	2	54	63	53	115	Died		+19	+17	+2
0.015(80'')*	1	28	48	55	55	140	Died		-3	+12	+4
0.017*	0	36	43	53	53	170	Died		+22	+10	0

Three dogs with Nembutal. Cold applied after adrenalin injection

0.010	5	7	39	50	52	150	48	36	+1	+18	-1
0.018(114'')	1	54	52	64	64	120	Died		+1	+9	-1
0.014	0	38	55	65	65	90	Died		+1	+22	-1

* Nembutal anesthesia given.

TABLE II

Percentage of water in the tissues taken from normal animals and from animals in adrenalin shock

The figures in parentheses represent the number of animals. The Fisher "t test" was employed for the statistical analysis of the data.

	Heart	Liver	Spleen	Bone	Testicle	Intes- tine	Chest muscle	Lung	Kidney	Leg muscle	Skin	Brain
Normal	78.1(12)	73.9(14)	77.6(14)	34.5(3)	84.5(4)	79.2(14)	74.1(13)	79.9(12)	81.4(15)	76.9(7)	59.4(13)	80.1(6)
Adrenalin shock	81.6(22)	75.6(22)	78.7(20)	40.7(3)	85.1(3)	79.5(20)	72.5(21)	78.8(21)	80.2(20)	76.0(9)	55.5(19)	76.7(3)
Difference	+3.5	+1.7	+1.1	+6.2	+0.6	+0.3	-1.6	-1.1	-1.2	-0.9	-3.9	-3.4
Statistically signifi- cant ($P \leq 0.05$)	+	+	+	-	-	-	+	-	+	-	-	-

injection are included here. There is an initial rise in the blood pressure when the adrenalin injection is started which is followed by a marked fall. In general, the drop in blood pressure continues until the animal expires. Similar patterns have been reported (5, 2) in arterial blood pressure as a result of sufficiently large doses of adrenalin.

Spectrographic analysis of serum potassium and sodium showed no consistent variation, while serum magnesium showed an average increase of 17 per cent in the 11 anesthetized animals examined. This has been noted as a rather consistent effect of Nembutal in many subsequent experiments and has no relation to adrenalin injection. The latter does not inhibit this effect.

A summary of the data concerning the percentage of water in the tissues in both normal controls and animals subjected to adrenalin shock is given in Table II. Since there was no apparent difference between the animals given Nembutal and those not given anesthesia, they have been grouped together. In the adrenalin experiments, the heart is subjected to an enormous strain, pericardial effusion usually occurs, and histological sections show some edema of the muscle. This evidence of increased fluid in the muscle is substantiated by an average increase of 3.5 per cent over the average normal water percentage determined by wet and dry weights in a rather large series of dogs. This increase in water content of the heart is highly significant when treated statistically. The Fisher "t test" was employed for the statistical analysis of the data, and a P value of 0.05 or less has been considered significant in this work. In the case of the heart, the P value was less than 0.01. By the same method, the liver and spleen show an increased total water content of 1.7 and 1.1

per cent, respectively. These increases are statistically significant. Samples of bone were taken from 3 control dogs and 3 adrenalin-shock dogs, and the average increase was 6.2 per cent in the adrenalin-treated animals. This gain, however, was not significant statistically, and is only suggestive in that the values in the different dogs varied over a rather wide range, and the number of animals studied was small. Of the tissues examined, only the chest muscle and kidney were found to have a significant decrease in water content as a result of adrenalin shock. The data suggest that the brain may lose water, but more animals must be investigated before this can be made certain. The average decrease in water content of skin as a result of adrenalin shock was 3.9 per cent, but this was not significant statistically. No consistent wet and dry weight measurements were obtained on the skin, probably because the amount of adherent fat, hair, and fascia varies widely. The fluid loss or gains as indicated by the water content of the tissues do not suggest that any large shift of water from the circulation has occurred in any one place, but rather that the change must be a diffuse one.

SUMMARY AND CONCLUSIONS

(1) Nembutal anesthesia results in a generalized parallel fall in the subcutaneous, intramuscular, and rectal temperatures of the dog.

(2) Injection of adrenalin under the conditions described has no effect on the rectal temperature. The peripheral muscle temperature drop is accentuated.

(3) On the basis of survival time, Nembutal anesthesia appears to have a deleterious effect in adrenalin shock.

(4) Applications of heat to the 4 extremities, in attempt to raise the peripheral temperatures

to normal, did not result in a beneficial effect in adrenalin shock.

(5) In general, the hematocrit was elevated as a result of the injection of adrenalin.

(6) The initial rise in arterial blood pressure when the adrenalin injection is begun is followed by a fall which continues fairly steadily until the animal succumbs.

(7) The most significant finding in the change in percentage of water in the tissues as a result of adrenalin shock was an increase in water content of the heart. Pericardial effusion usually occurs, and edema of the heart was also demonstrated histologically. The quantity of water involved is of no significance to the total water balance.

(8) The findings indicate that, in this type of shock, there is no specific mobilization of water in the tissues which accounts for the rather frequent finding of a rise in hematocrit (hemocentration).

(9) Serum potassium and sodium showed no

consistent changes. A rather consistent increase (average of 17 per cent) in serum magnesium was found in those dogs given Nembutal anesthesia and was attributed to the latter.

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A STUDY OF THE AGGLUTININ RESPONSE IN PATIENTS WITH MENINGOCOCCIC MENINGITIS¹

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Although many workers have discussed the development of humoral antibodies in patients with meningococcic infections, the results of their observations have been irregular and at times conflicting. It has recently been concluded (1) that, of all the methods available at present, the agglutination test is the most reliable. These workers were unable to achieve high agglutinin titers by the use of the usual method of incubation for 2 hours at 37.5° C., followed by 18 hours in the refrigerator, but with the use of the "thread test" they obtained consistent titers ranging up to 1:128 in patients with meningococcic meningitis and as high as 1:64 in contacts. We have likewise not observed sufficiently high titers with the orthodox method of incubation followed by refrigeration, and since the "thread test" is difficult to perform, except by persons highly trained in its use, we looked for another method which would be both reliable and practicable for routine use.

In the course of our search, we found that the use of centrifugation, in place of heating and refrigeration, gave consistently high titers on the sera of most patients during the course of meningococcic meningitis. The test is easy to perform and the results are highly specific. Sera obtained from patients with a variety of other diseases, including pneumococcic pneumonia and meningitis, gonococcic cervicitis, arthritis and meningitis and streptococcus viridans endocarditis, usually showed no agglutinins at all. In the occasional case where they did appear, the agglutinin titer was 1:2, 1:4, or, in one instance, 1:8. One patient with lymphocytic choriomeningitis exhibited an agglutinin titer of 1:64 on the fourth day of her illness and titers

of 1:32 in all of the 5 other specimens of sera examined, the last one having been obtained on the fourteenth day of the disease. We are unable to account for these titers, which were so much higher than any others found in patients with infections not caused by the meningococcus, unless the patient might possibly have been a carrier of meningococci. This concept is strengthened by the fact that she was admitted during the peak of the meningitis epidemic, but cannot be proved, since no cultures were made from her throat.

The present report is a correlation between the results of our centrifugation-agglutination test and the course of the disease in patients with meningococcic infections.

PROCEDURE

Specimens of serum were collected during the course of the disease, usually at intervals of 1 or 2 days, from the day of admission until the day the patient was discharged from the hospital. In occasional patients, only 1 or 2 samples of serum were obtained.

Altogether 212 samples of serum were collected from 47 patients with typed meningococcic meningitis, and 70 specimens of serum from 20 patients who had meningitis resembling meningococcic meningitis clinically but from whose spinal fluid meningococci could not be cultured or typed by the immediate "quellung" method, although in some instances gram-negative diplococci were seen. A single agglutinin test was also done on each of 2 patients who appeared to have meningococcemia clinically, although the organisms were never cultured from the blood. All patients received sulfadiazine or sulfamerazine and 2 of them received penicillin.

The details of the technique of this test will be published elsewhere (2). Briefly, it was carried out as follows: Suspensions of strains of group I and group II alpha meningococci, freshly isolated from patients with the disease, were killed at 65° C. and suspended in physiological salt solution without preservative. Serial dilutions of the patient's serum were prepared, and an equal amount of the diluted, killed suspension added to each tube, as in the usual agglutination test. Instead of incubation and

¹ This study was aided by a grant from the Lederle Laboratories, Inc.

TABLE I

Agglutinin titers achieved in cases of group I meningococcic meningitis

Titer	Day of disease								All cases
	1 to 5	6 to 10	11 to 15	16 to 20	21 to 25	26 to 30	30 and over	Date of onset not known	
0	37	8	3	6	3	3	9	2	71
1:2	1	1	1	1	0	0	1	0	5
1:4	3	3	1	4	0	0	0	0	11
1:8	0	1	4	0	1	0	0	0	6
1:16	4	1	1	0	0	0	0	0	6
1:32	1	6	2	0	0	0	0	0	9
1:64	7	4	2	4	1	0	0	1	19
1:128	5	9	10	1	1	0	0	1	27
1:256	2	11	11	6	1	1	0	0	32
1:512	0	0	1	1	0	0	0	0	2
Total	60	44	36	23	7	4	10	4	188

refrigeration, however, the tubes were immediately centrifuged at 2,000 revolutions per minute for 20 minutes, after which the agglutinin titer could be read immediately.

RESULTS

Table I shows the agglutinin titers found on the 188 specimens of serum from the 44 patients with meningitis caused by group I meningococci to whom no antimeningococcic serum was given. All of the titers shown in the table were obtained with suspensions of group I meningococci, while a titer of zero was obtained with suspensions of group II alpha meningococci. In the case of 1 patient, not shown in the table, the meningococci found in the spinal fluid were typed as group I, while a single specimen of serum taken on the twentieth day of the disease was positive in a titer of 1:1024 for group II alpha and negative for group I.

It will be seen that during the first 5-day period after the onset of the meningitis the agglutinin titer was zero in the majority of instances. The titer for most patients rose considerably during the next 2 periods, being 1:64 or above in the majority of cases from the sixth through the tenth day and 1:128 or above from the eleventh through the fifteenth day. Thereafter, the titer usually fell rather rapidly. In the period between the sixteenth and twentieth days, in about half of the cases, the titer was 1:4 or less, while in the other half, the titer was still high, 1:64 to 1:512. Except for 1 titer of 1:256 on the thirtieth day of the disease and 1 titer of 1:2 on the thirty-fifth day, all agglutinin titers had

returned to zero after the twenty-fifth day, and in these 2 cases a specimen of serum taken still later gave an agglutinin titer of zero.

In Figure 1 the median agglutinin titers for each 5-day period are shown by solid circles, while the solid line represents the smoothed curve of the values which were obtained in all the patients with group I meningococcic meningitis in whom the day of the onset of the disease was known. The initial spinal fluid in all these patients was examined before treatment was begun, and the number of meningococci was counted in the average high-power field in a stained smear of the sediment after centrifugation. If there were an average of 5 or more organisms per high-power field, these were

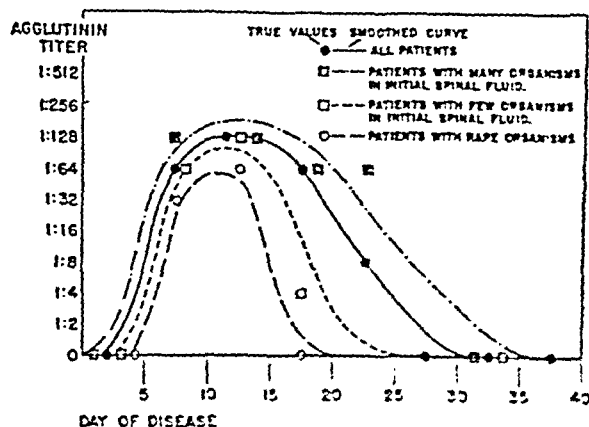


FIG. 1. MEDIAN AGGLUTININ TITERS IN PATIENTS WITH GROUP I MENINGOCOCCIC MENINGITIS, ARRANGED BY 5-DAY PERIODS FROM DATE OF ONSET

designated as "many" organisms. When 1 to 4 diplococci were present, they were considered to be "few" in number, and if less than 1 diplococcus per high-power field was seen, organisms were said to be "rare." From the figure it will be seen that, when "many" organisms were present in the initial spinal fluid, the agglutinin titers in the patient's serum usually rose to higher levels and fell more slowly than when only a "few" organisms were present. When organisms were "rare" in the initial specimen, the maximal titer achieved was usually lower than in any of the other groups and the titer fell to zero sooner.

We have not been able to correlate the agglutinin titers achieved with any other factor with which we have compared them, such as, the age of the patient, the presence or absence of a positive blood culture, the presence of complications of the disease, the day of the disease on which specific treatment was begun, or the rapidity of response to specific treatment. Agglutinin titers were determined on 4 patients who subsequently died and who had group I meningococci in the spinal fluid. One of these had an agglutinin titer of 1:32 on the sixth day of the disease and died on the eighth day. The second had a titer of 1:64 on the fifth day and died on the seventh day. The third came in in coma so that the date of onset of his illness could not be ascertained. He had an agglutinin titer of 1:128 on the third day of his hospital stay and died on the following day. The fourth patient was also unable to give a history. He had no agglutinins on the second and third days of hospitalization and died on the third day. Apparently the height of the agglutinin titer is not related to the outcome of the disease, at least in patients receiving chemotherapy.

One patient, not included in Table I, had no agglutinins on the fourth day of his disease. Because he was seriously ill, he was given 80 ml. of polyvalent antimeningococcic serum the same day. One hour later he had a titer of 1:64 for group I meningococci and none for II alpha. Eleven days later the titer for group I was 1:128 and thereafter it gradually descended to 1:8 on the fifty-third day of the disease, when he was discharged.

Specimens of serum for agglutination were obtained on 1 patient whose spinal fluid was found to contain group II alpha meningococci. He was admitted on the fourth day of the disease. Thirteen specimens of serum were obtained. Agglutinin titers with a suspension of group II alpha meningococci started at zero on the fourth day of his illness, slowly rose to 1:64 on the eleventh day, and fell to a titer of 1:4 on the nineteenth day when he was discharged from the hospital completely recovered. All agglutinin controls for group I meningococci were negative.

It is of interest that among the 20 patients on whom 5 or more specimens of serum were tested, all had some agglutinins at one time or another. In 1 patient, the highest titer was 1:2; in another, it was 1:4; in 2 more, 1:8; while in all the others, the highest titer was 1:32 or more.

TABLE II

Agglutinin titers in patients with meningitis clinically resembling meningococcic meningitis or meningococcemia but from whom no meningococci were obtained

Agglutinin titer	Tests on patients with meningitis		Tests on patients with apparent meningococcemia
	Organisms seen on smear	No organisms seen	
0	23*	19	0
1:2	3*	3	0
1:4	2	1	1
1:8	2	0	0
1:16	0	2	0
1:32	1	3	0
1:64	0	1	0
1:128	1	3	1
1:256	1	0	0
1:512	1	2	0
Total number of tests	34	34	2

* Agglutinin titers all for group I (and zero for group IIa) except in the case of one patient where they were positive for group IIa and zero for group I.

In Table II are shown the agglutinin titers in patients who had conditions clinically resembling meningococcic meningitis, or meningococcemia, although no organisms were cultured or typed from the spinal fluid or blood. In the second column are shown the tests done on patients with meningitis in whose spinal fluid gram-negative diplococci were seen, and in the third column, those done on patients whose spinal

fluid revealed no organisms. In the fourth column are 2 tests done on the serum of patients with a disease clinically resembling meningococcemia whose blood cultures were negative. It will be seen that agglutinin titers at all levels up to 1:512 were obtained in these cases, and that there was no significant difference between the titers, whether organisms were seen in the spinal fluid or not. When a comparison is made with Table I, it is seen that there was a tendency for higher agglutinin titers to occur in the sera of patients from whom meningococci were cultured. Although the number of cases was not great enough to permit a valid comparison, we found that the titers in the patients who showed no organisms on smear and culture corresponded closely with the titers in the patients with rare or no organisms on smear but with positive cultures.

DISCUSSION

Because there has been no complete study of the rise, duration, and fall of agglutinin titers during the course of meningococcic meningitis, we have undertaken such a study by testing sera from 67 patients with this disease and 2 patients with an infection clinically resembling meningococcemia. Group I or group II alpha meningococci were typed from the spinal fluid of 47 of these patients. Organisms morphologically resembling meningococci were seen in the spinal fluid of 10 additional patients, while in 10 patients, the disease was diagnosed on clinical grounds alone. The agglutinins were specific for the group of meningococcus typed from the spinal fluid, except in 1 instance in which group I meningococci were found, while a single specimen of serum on the twentieth day of the disease showed an agglutinin titer of 1:1024 for group II alpha and none for group I. We are unable to account for this discrepancy. Since the agglutination test was done several weeks after the patient was discharged from the hospital, we could not repeat the typing or secure other samples for agglutination. The agglutination test was clear-cut in its results, so that, if a mistake was made, it may have been in the typing at the time of admission or in recording the results of the typing.

When we considered only the patients with

typed meningococcic meningitis in whom the day of onset was known, we found that the median agglutinin titer rose from zero in the first 5-day period after the onset of the meningitis to 1:64 and 1:128 in the second and third 5-day periods, respectively. Thereafter the median titer fell until it reached zero between the twenty-fifth and thirtieth day, although in individual cases the titers remained elevated until as late as the fifty-third day of the disease. If we were to add the titers for the untyped cases to those of the typed group, the median values for all cases would remain the same.

In a recent study (3) of agglutinins in patients with meningococcic meningitis, the first significant titers (1:30 or more by their technique) were found to occur on the seventh day of the disease and the last ones on the forty-ninth day. Titers went as high as 1:240 during the peak period, which was stated to be around the twenty-first day. Our results were similar to these except that the titers we obtained were higher in general, presumably due to the fact that the previous investigators used the usual heat-followed-by-refrigeration method. In the few cases which Thomas and his associates (1) report, the trend of agglutinin titers is also comparable with those that we have encountered.

In general, agglutinins were found to rise to higher levels the greater the number of meningococci found in the initial specimen of spinal fluid, and to be lowest when only a rare organism was present at the time of the first spinal puncture. It is interesting in this connection that the chance of recovery has been shown (4) to be inversely proportional to the number of organisms in this initial specimen of spinal fluid. Since only 4 patients in the present group died, the agglutinin titer could not be related to prognosis, although it was observed that 3 of these patients had agglutinins in significantly high titers before death. The agglutinin response could not be correlated with any other factors that we were able to study.

In view of the fact that most of our patients with meningitis showed a high titer of agglutinins at some time during the course of the disease, if a sufficient number of specimens were examined, while, on the other hand, only 1 of the patients with other infectious diseases showed a

titer above 1:8, this test is obviously useful in detecting the presence of meningococcic infections when organisms cannot be typed from the blood or spinal fluid. Certain investigators (5) have diagnosed meningococcemia from the clinical features of their cases plus the presence of agglutinins as demonstrated by the "thread test." Since we have obtained a significantly high titer in one case of clinical meningococcemia and a suggestive titer in another, we would recommend that the centrifuge-agglutination test be employed when meningococcemia is suspected. From present evidence, titers above 1:64 or rising titers would confirm the clinical diagnosis of a meningococcic infection.

SUMMARY AND CONCLUSIONS

1. Two hundred and eighty-four specimens of serum from 69 patients with meningococcic meningitis or meningococcemia were examined for agglutinins by means of a test using centrifugation instead of the usual method of heating at 37.5° C. followed by refrigeration.

2. Agglutinin titers were specific for the group of meningococcus isolated from the spinal fluid of the patient except in one instance. The titers ranged from zero to 1:1024. The median titer in typed group I patients rose to

1:128 for the period from the eleventh to the fifteenth day, inclusive, and fell to zero in the period between the twenty-fifth and thirtieth days.

3. The median agglutinin titer rose to higher levels the greater the number of organisms present in the initial specimen of spinal fluid.

4. The centrifugation-agglutination test is a simple, rapid, and efficient method which may be used to diagnose cases of meningococcic infections where organisms cannot be cultured from the body fluids.

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BACTERIOSTATIC AND LYTIC ACTIONS OF PENICILLIN ON SENSITIVE AND RESISTANT STAPHYLOCOCCI¹

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(Received for publication July 17, 1944)

In contrast to other bacteria, staphylococci undergo lysis when allowed to multiply in the presence of bacteriostatic concentrations of penicillin. As noted previously (1), all penicillin sensitive strains are lysed, whereas insensitive strains resist this lytic action. The following observations clarify certain fundamental aspects of both lysis and bacteriostasis, and have important clinical as well as theoretical implications.

EXPERIMENTAL

The general methods have been described in detail elsewhere (1). For the present study, large inocula (10 to 50 million organisms per ml.) of 100 penicillin-sensitive strains of *Staph. aureus* (coagulase positive), isolated from patients with furuncles, abscesses, or osteomyelitis, were

¹ The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutics and Other Agents of the National Research Council.

added to tubes containing synthetic medium plus the following concentrations of penicillin, 0.1, 1.0, 10, and 100 U. per ml., and growth was measured turbidimetrically at various intervals for from 3 to 7 days. With 10 of the strains, plate counts were made from each tube daily for 3 days, and further tests of viability of the organisms were carried out after 7 days.

In addition, 8 strains of penicillin-resistant staphylococci obtained from clinical sources were tested by the serial dilution technic (2), as well as by the above method, in an attempt to correlate resistance with lysis and bacteriostasis.

RESULTS

A typical lysis experiment is shown graphically in Figure 1. With 0.1 U. per ml. of penicillin there was greater initial growth than with the higher concentrations. This was followed by rapid lysis, so that, after 24 hours, the tube containing 0.1 U. per ml. was much less turbid than those with 1.0, 10, and 100 U. per ml. of penicillin. This phenomenon, which occurred with each of the 100 strains, was so striking that

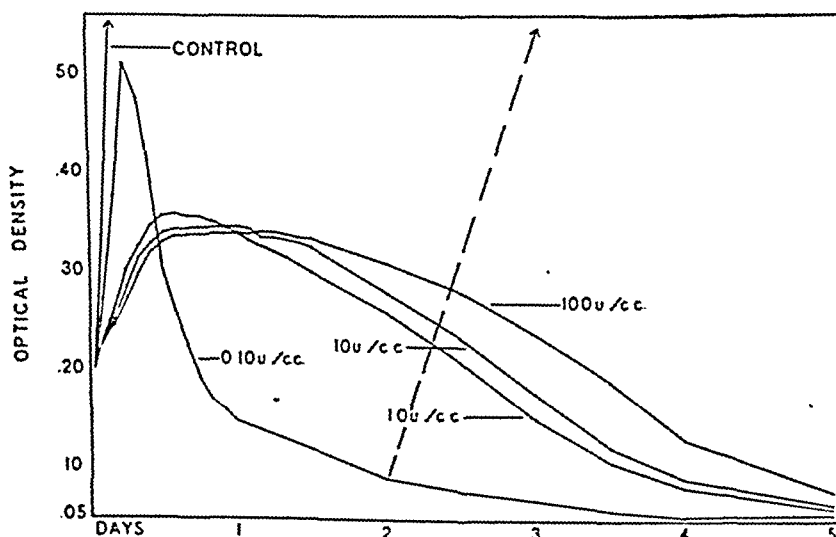


FIG. 1. GROWTH AND LYSIS OF A STRAIN OF *Staphylococcus aureus* MEASURED TURBIDIMETRICALLY

The correlation between penicillin concentration and degree and rate of lysis is clearly demonstrated.

TABLE I

Results of plate counts in a typical lysis experiment

Fewer viable organisms were present with 0.1 U. per ml. than with 100 U. per ml. of penicillin. X = too many colonies to count accurately.

	10^{-2}	10^{-4}	10^{-6}	10^{-8}
24 hours				
Control	X	X	X	810
0.1 U. per ml.	X	X	200	28
100 U. per ml.	X	X	684	79
48 hours				
Control	X	X	X	540
0.1 U. per ml.	X	42	3	0
100 U. per ml.	X	600	43	2
72 hours				
Control	X	X	X	500
0.1 U. per ml.	21	16	1	0
100 U. per ml.	594	40	20	0

it could be readily observed grossly by holding the tubes up to the light. After 24 hours, there was gradual lysis in all the tubes containing penicillin, the rate now faster with the higher concentrations, so that after 5 days, turbidities in all the tubes were about the same.

Lysis was closely correlated with destruction of bacteria. Many more viable organisms were present after 24 hours with 100 U. per ml. than with 0.1 U. per ml., and during the next 72

hours, there was a decrease with both concentrations, the count falling rapidly with 100 U. per ml. as lysis occurred. Results of plate counts with a representative strain are presented in Table I. Even after a week, viable organisms could always be recovered from each tube, but this prolonged incubation was in no instance associated with a demonstrable development of penicillin resistance.

With a very heavy inoculum (optical density 0.4), similar results were obtained (Figure 2). Lysis occurred more slowly, but its relation to the concentration of penicillin was the same as with the smaller inoculum.

With 3 strains obtained from blood cultures, there was a secondary growth in the tube containing 0.1 U. per ml. (represented by a broken line in Figure 1). This occurred after 48 hours' incubation; *i.e.*, after a large percentage of the organisms had undergone rapid lysis. The staphylococci now present were highly resistant, growing luxuriantly without lysis in the presence of 100 U. per ml. of penicillin.

These resistant organisms, plus 5 naturally occurring resistant strains, all responded to the above lysis test in the same manner; a typical example is shown in Figure 3. No lysis oc-

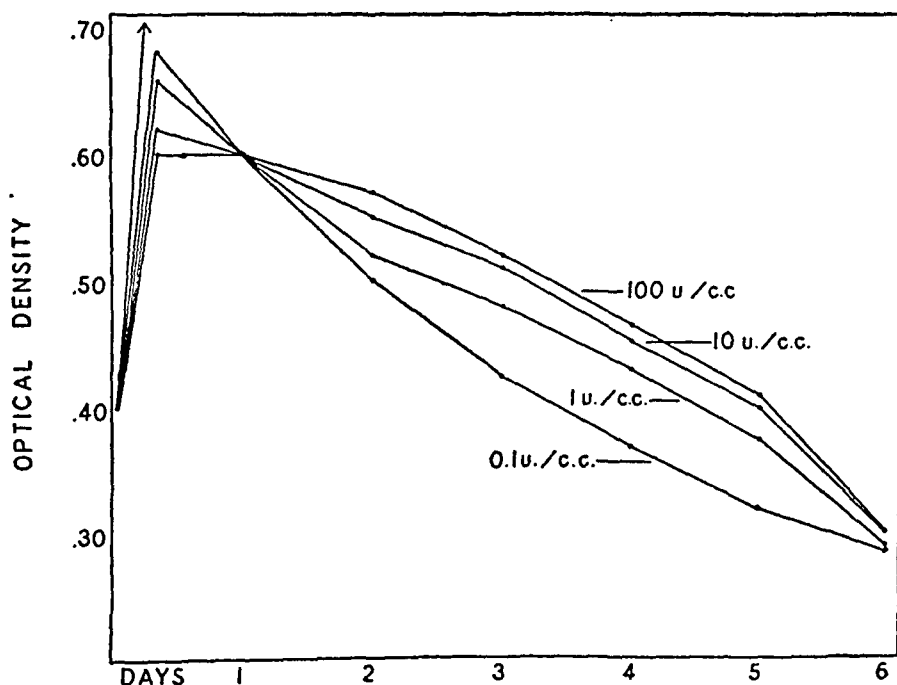
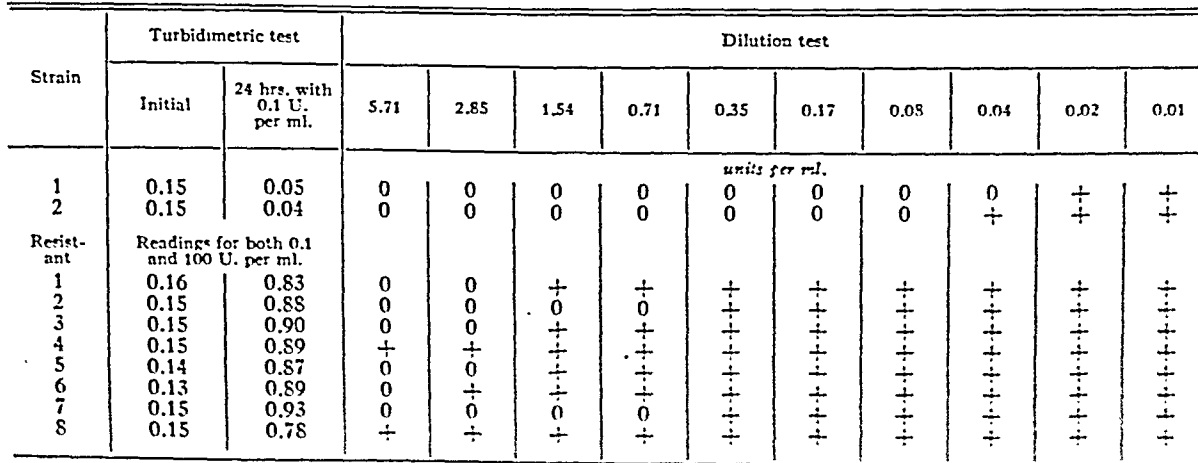


FIG. 2. THE SAME AS FIG. 1, USING A MUCH LARGER INOCULUM

Lysis occurred more slowly, but the relation to penicillin concentration was the same as with the smaller inoculum.



curred more rapidly with smaller than with larger concentrations, presumably because of the greater initial growth of the organisms with smaller concentrations. In other words, the greater the initial growth of the organisms in the presence of a bacteriostatic concentration of penicillin, the more rapid their destruction. It is of interest that the concentration of penicillin causing the most rapid lysis, 0.1 U. per ml., is in the range obtained clinically in the blood stream, especially with the continuous intravenous method of administration. A further point of clinical interest is the effect of the lysed cellular elements upon the patient. The prolonged high fever and toxicity in cases of staphylococcus sepsis as opposed to those caused by pneumococci or hemolytic streptococci may be in part due to substances liberated by the lysis of large numbers of staphylococci.

The outgrowth of highly resistant staphylococci in 0.1 U. per ml. of penicillin suggests the presence in the culture of a few resistant bacteria which could not multiply rapidly until the majority of the sensitive organisms had been lysed. This suggestion is supported by the observation of the Oxford group that artificially induced resistance of staphylococci is associated with a diminished velocity of growth (3). Virulence of the resistant organisms has not been investigated. The clinical implications of this observation are not clear, but it is worth noting that, in one instance, the secondary outgrowth of resistant staphylococci occurred in the culture from a patient with osteomyelitis whose response to penicillin was unsatisfactory.

These resistant staphylococci have been found to produce an intracellular penicillin inactivator (4) in contrast to sensitive strains which produced no such penicillin-destroying substance. The properties of this penicillin inhibitor have been described (5) and it has been added to routine culture media to inactivate penicillin present in specimens obtained from patients under treatment (6).

In the present experiments with resistant staphylococci, resistance to lysis and resistance to bacteriostasis have been clearly differentiated. In Figure 3, for example, penicillin exerted a marked bacteriostatic action on the organisms, although lysis did not occur. At the end of 24

hours, growth in the presence of penicillin was equal to that of the controls; a single observation made at this time would have conveyed the impression that this strain of *S. aureus* was "completely resistant" to the bacteriostatic action of penicillin, when such was actually not the case. The dilution tests, summarized in Table III, demonstrated a further important point in regard to the size of the inoculum. With a small inoculum (1000 to 30,000 organisms per ml.), viable organisms could not be recovered in most instances after 24 hours with penicillin concentrations of 5 U. per ml. or less, whereas there was luxuriant growth in the presence of 100 U. per ml. with the large inoculum (10 million organisms per ml.) used for the turbidimetric test. This was apparently due to the inability of the smaller number of bacteria to multiply a sufficient number of times to destroy even relatively small concentrations of penicillin. It is evident then that resistance is a relative matter. Resistant organisms produce a penicillin destroying substance, but are themselves susceptible to the bacteriostatic action of penicillin, and will not survive unless they are present in sufficient numbers to inactivate the penicillin with which they are in contact. This concept should be borne in mind in performing and interpreting tests designed to determine the sensitivity of staphylococci to penicillin.

The importance of the size of the inoculum is also demonstrated by the experiments with sensitive staphylococci. With the large inoculum used for the turbidimetric tests, viable organisms were recovered after 7 days' incubation in the presence of 100 U. per ml., while the small number of bacteria employed for the dilution test were killed within 24 hours by less than 0.1 U. per ml. Thus the statement that the action of penicillin is "only influenced to a minor extent by the number of bacteria to be inhibited" (3) must be accepted with definite reservations.

SUMMARY

1. *In vitro* studies of the action of penicillin on 100 sensitive and 8 resistant strains of *S. aureus* are reported.

2. A striking correlation was found between the concentration of penicillin and the degree and rate of lysis of sensitive organisms.

3. In 3 instances, there was a secondary outgrowth of highly resistant staphylococci following the initial rapid lysis of sensitive organisms.

4. Penicillin-resistant organisms were susceptible to the bacteriostatic action of penicillin, but overcame this action by destroying the penicillin when present in sufficient numbers. When present in insufficient numbers, they succumbed to the action of penicillin in the same manner as sensitive staphylococci.

5. The size of the inoculum was found to have an important bearing on the results of sensitivity tests, both with sensitive and resistant staphylococci.

I am indebted to Mrs. Mary Wolohan for technical assistance.

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PROPERTIES OF A PENICILLIN INACTIVATOR EXTRACTED FROM PENICILLIN-RESISTANT STAPHYLOCOCCI¹

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An enzyme-like substance capable of destroying penicillin was produced from *E. coli* and certain other saprophytic and commensal bacteria in 1940 (1), but attempts to extract a penicillin inactivator from sensitive or resistant staphylococci were unsuccessful (2). More recently, acetone-ether extracts of para-colon bacilli which were more effective penicillin inhibitors than were extracts of *E. coli* were prepared (3).

In this country, it was found (4) that Clarase, a diastatic enzyme preparation, readily destroyed penicillin, and this substance is now used widely for sterility tests during penicillin production. Apparently, the ability to inactivate penicillin is possessed by only certain lots of Clarase (5), and this action is attributed in a later publication (6) to the presence of bacterial products, especially of *B. subtilis* and related gram-positive organisms.

A penicillin-destroying staphylococcus has been found contaminating a culture of *Aspergillus flavus* (7), and independently, in this laboratory, a highly potent penicillin inactivator has been extracted from penicillin-resistant staphylococci obtained from clinical sources (8). Details of the extraction and properties of this substance are presented in the present paper.

DESCRIPTION OF RESISTANT STAPHYLOCOCCI

During a study of several hundred strains of staphylococci isolated from patients (9, 10), a few were found which were completely resistant to the lytic action of penicillin; 5 of these were selected for the present study. Two additional strains were cultured from a secondary outgrowth of highly resistant staphylococci following

the lysis of susceptible organisms by 0.1 U. per ml. of penicillin (9). All 7 strains produced yellow pigment, and there were no abnormalities of growth or colony characteristics. Details of the sensitivity tests have been described elsewhere (9, 10).

METHOD OF EXTRACTION

The acetone-ether method of Harper (3), devised for the para-colon bacillus, was equally satisfactory for the staphylococcus. Twenty-four-hour plate cultures were washed with a minimum of saline and precipitated with 7 volumes of acetone. After standing 2 hours, the precipitate was treated with a fresh supply of acetone for an hour, washed with 2 changes of ether, and dried in the anaerobic jar. During extraction, the coarse precipitate was crushed repeatedly with a glass rod to insure maximum contact between the acetone and the organisms. The fine dried powder was stored in a desiccator at room temperature.

MEASUREMENT OF PENICILLIN INACTIVATION

The powder was suspended in broth, usually in a concentration of 1 mgm. per ml. The ability of various amounts of this suspension to inactivate penicillin was measured by recording the growth of a Group A hemolytic streptococcus which was inhibited by less than 0.05 U. per ml. of penicillin. To tubes containing the powder and a heavy inoculum (about one million organisms per ml.) of the test bacterium, penicillin was added in concentrations of 0.1, 1.0, 5.0, and 10.0 U. per ml., and optical densities (turbidities) were measured every few hours on a Coleman universal spectrophotometer during 24 hours, incubation. The exact amounts of broth, penicillin, etc.,

TABLE I
Outline of test for measurement of penicillin inactivation

Tube	Broth plus hemolytic streptococcus	Penicillin	Penicillinase	Sterile broth
	ml.	1.0 U. per ml.	1 mgm. per ml.	
1	10	0	0	0
2	9	1 ml.	0	0
3	8	1 ml.	1 ml.	0
4	8	1 ml.	1 ml.	0
5	8	1 ml.	1 ml.	0
6	8	1 ml.	1 ml.	0
7	0	0	1 ml.	9

¹ The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for experimental investigations recommended by the Committee on Chemotherapeutics and Other Agents of the National Research Council.

are listed in Table I. The results of a typical experiment are presented in Figure 1.

Penicillin was destroyed gradually, with the length of time required for complete destruction roughly proportional to the amount of penicillin present. One hundred units were completely destroyed by 1 mgm. of the powder within 6 to 8 hours. By decreasing the amount of powder, it was found that 0.6 mgm. would cause complete destruction of 100 units of penicillin in 24 hours, whereas 0.4 mgm. would not (Figure 2).

Using this arbitrary endpoint, it could be said that 1 mgm. of the powder would destroy 167 units within 24 hours. Although there were some variations, this same high degree of potency was shown by the extracts of all 7 resistant strains.

Penicillin sensitive staphylococci were extracted and tested in the same manner. Extracts of 7 strains, isolated from blood cultures of patients with acute infections, all failed to cause any demonstrable inactivation of penicillin, even with 2 mgm. of the powder and only 1 unit of penicillin.

PROPERTIES OF THE PENICILLIN-DESTROYING SUBSTANCE

Heat stability. Broth suspensions of the powder could be kept in the incubator (37° C.) for at least 48 hours, or at room temperature for several weeks with no detectable diminution in their ability to destroy penicillin. At 56° C., however, the ability to inactivate penicillin was completely lost in 5 minutes or less.

Rate of destruction of penicillin at different temperatures. Broth suspensions of the powder (1 mgm.) and penicillin (100 units) were left at 2° C., 22° C., and 37° C. for 2, 3, 4, and 5 hours, and then tested for penicillin destruction in the usual manner. In every instance, growth of the test organism occurred at the same rate for the mixtures left at 2° C., 22° C., and 37° C. after any given time interval, indicating that penicillin was destroyed as rapidly at 2° C. as it was at room temperature or in the incubator.

Nature of penicillin inactivation. The following experiment was performed to determine whether penicillin was actually destroyed, or whether it merely entered into a chemical com-

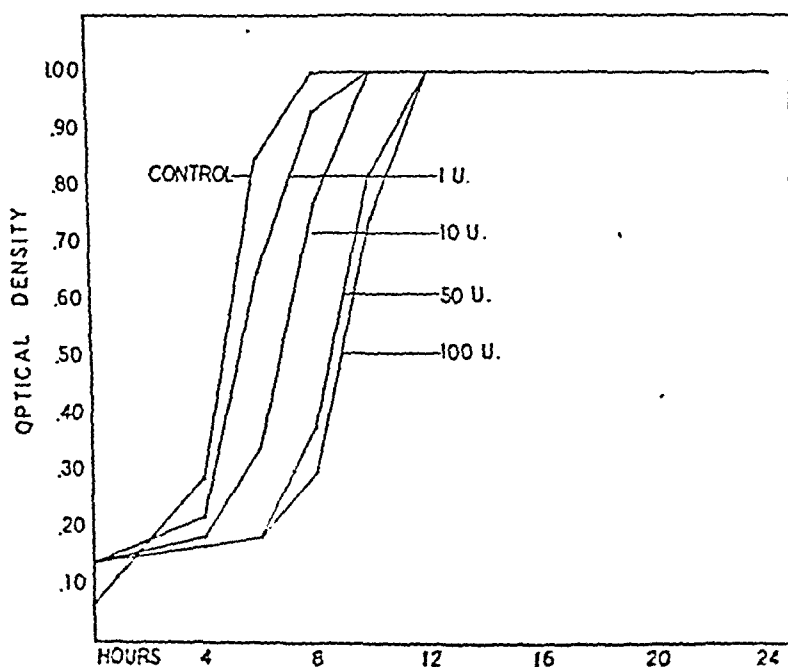


FIG. 1. DESTRUCTION OF VARIOUS AMOUNTS OF PENICILLIN BY 1 MGm. OF THE STAPHYLOCOCCUS EXTRACT

Complete destruction is indicated by growth of the organism which was inhibited by 0.05 U. per. ml. of penicillin.

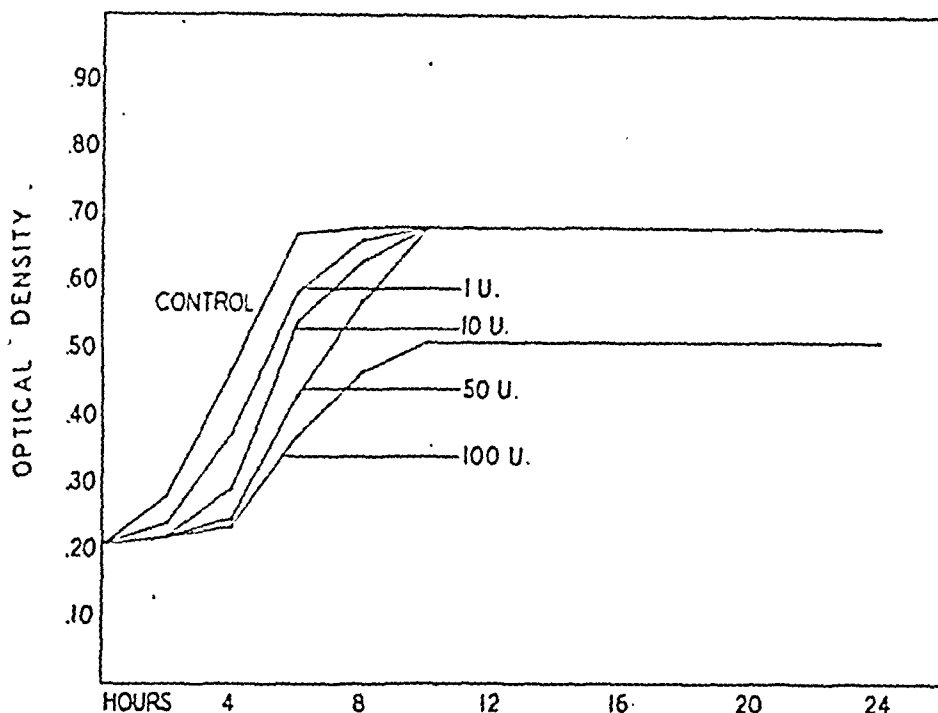


FIG. 2. INACTIVATION OF PENICILLIN BY 0.4 MG. OF THE DRIED POWDER

Fifty units were destroyed, whereas 100 were not, giving an arbitrary endpoint for titrating the potency of the powder.

bination with some constituent of the bacterial extract. One mgm. of the dried powder was added to 100 units of penicillin, and left at room temperature overnight. The next morning the mixture was placed in a 56° C. waterbath for 15 minutes to destroy the penicillin inactivator. The solution was then tested for penicillin, and none could be demonstrated. Fifteen minutes at 56° C. caused no loss of potency of a control sample of penicillin. This suggests that the penicillin was actually destroyed, although the possibility of a chemical combination not disrupted by heat cannot be definitely excluded. The observation that penicillin could not be extracted with amyl acetate after inactivation by penicillinase is further evidence in favor of actual penicillin destruction (3).

Penicillin inactivation by living cultures. Growth of resistant staphylococci in the presence of penicillin was associated with destruction of the penicillin. During this destruction, definite bacteriostasis occurred but the organisms resisted lysis and multiplied so that after 24 hours, growth was equal to that of controls containing no penicillin. This is illustrated in Table II. Complete destruction of penicillin

was confirmed by testing Seitz filtrates of the culture fluid for penicillin; none was present.

Seitz filtrates of cultures of penicillin-resistant staphylococci or of broth suspensions of the powdered extract did not destroy penicillin; *i.e.*, the penicillin was intracellular, and did not pass out into the culture fluid.

COMPARISON WITH CLARASE

In contrast to the staphylococcus extract, Clarase (lot. No. 1351) was dissolved in phosphate buffer at pH 7, and the penicillin inactiva-

TABLE II

Turbidimetrically recorded growth of a penicillin-resistant S. aureus

Although there was definite bacteriostasis, the penicillin was destroyed and growth after 24 hours was equal to that of the control. Turbidity is expressed in terms of optical density.

Penicillin	Hours			
	0	4	6	24
0	0.168	0.57	0.745	0.77
10 U. per ml.	0.168	0.376	0.52	0.77
100 U. per ml.	0.168	0.285	0.376	0.77

tor passed readily through a Seitz filter. Ability to destroy penicillin was tested in the manner described for the dried powder. Using a 1 per cent solution of Clarase, destruction of 100 units of penicillin by 0.6 ml. (6 mgm.) was equivalent to 0.6 mgm. of the staphylococcus extract; *i.e.*, in terms of weight of the original powder, the staphylococcus extract was 10 times as potent as Clarase. With equivalent quantities, the rate of destruction of penicillin at 2° C., 22° C., and 37° C., was identical with both substances. Clarase was quite stable at room and incubator temperatures and was more stable at 56° C. than the staphylococcus extract, activity being only partially destroyed by exposure to that temperature for 30 minutes.

DISCUSSION

A highly potent penicillin inactivator has been extracted from 7 strains of *S. aureus* which were naturally resistant to penicillin; 7 sensitive strains did not produce a penicillin-destroying substance. The resistant bacteria were not lysed, but were by no means completely resistant to the bacteriostatic action of penicillin, as indicated in Table II. This differentiation between resistance to lysis and resistance to bacteriostasis is discussed elsewhere (10).

It is of fundamental importance to determine

whether the several penicillin inactivators so far described are identical, or whether penicillin can be destroyed by substances whose chemical structures are unrelated. Properties of the known penicillin inactivators are summarized in Table III. Presumably they are all products of bacterial metabolism, but whether they are produced by all penicillin-resistant organisms has not yet been definitely determined. The differences in properties are relatively superficial, and possibly largely due to differences in technical methods. Further chemical studies are necessary for final clarification.

Resistant staphylococcus extracts were 10 times as effective as Clarase in their ability to destroy penicillin, but since the potency of Clarase is presumably due to the presence of unknown amounts of bacterial products, no exact comparison could be made.

Penicillin inactivators are used for sterility tests, and for cultures from patients receiving penicillin. A brief description of the technic and results of the addition of staphylococcus extracts to culture media has been published elsewhere (11).

The presence of a penicillin inactivator in extracts of resistant but not of sensitive staphylococci is of interest in connection with the recent observation (12) that the development of sulfonamide-resistant strains of staphylococci is

TABLE III
Summary of the properties of the known penicillin inactivators

Source	Apparent ability to inactivate penicillin	Heat stability	Destruction of penicillin by living cultures	Presence of inactivator in culture fluid	Rate of penicillin destruction at 2° C., 22° C., and 37° C.
<i>E. coli</i> (Abraham and Chain)	+	Destroyed by 90° C. for 5 min.	Partial (Harper)	Absent	Slower at 25° C. than 37° C.
Unidentified gram-negative rod (Abraham and Chain)	+	Not known	Indefinite	Present	Not known
Paracolon (Harper)		No destruction at 50° C. for 30 min.	Complete	Present	Not known
Clarase (Lawrence)		Partial destruction at 56° C. for 30 min.		Present	Same with all temperatures
Staphylococcus (McKee, Rake, and Houck)	Indefinite	Not known	At least partial	Present	Not known
Staphylococcus (Kirby)		Complete destruction at 56° C. for 5 min.	Complete	Absent	Same with all temperatures

related quantitatively to the production of para-aminobenzoic acid. The evidence is too incomplete to suggest the possibility of competitive enzyme systems as a basis for the explanation of the mode of action of penicillin, but the analogy is striking. Unfortunately, staphylococci made resistant *in vitro* have not been tested in this laboratory for penicillin inactivation. There is a need for refined methods of extraction to search for the presence of penicillin inhibitors in sensitive staphylococci, as well as for intensive study of the chemical nature of the inhibitors themselves. It seems not unreasonable to suppose that, as with the sulfonamides, a more fundamental understanding of the mode of action of penicillin will come as a result of a study of its inhibitors.

SUMMARY

A penicillin inactivator has been extracted from 7 strains of penicillin resistant *Staphylococcus aureus* obtained from clinical sources; 7 sensitive strains did not produce a penicillin-destroying substance. Properties of the penicillin inactivator are described, and its relation to other inhibitors and to the mode of action of penicillin in general is considered.

I am indebted to Mrs. Mary Wolohan for technical assistance.

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AN EXPERIMENTAL ATTEMPT TO TRANSMIT PRIMARY ATYPICAL PNEUMONIA IN HUMAN VOLUNTEERS¹

By THE COMMISSION ON ACUTE RESPIRATORY DISEASES²

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INTRODUCTION

During the past 10 years, the clinical and roentgenographic features of primary atypical pneumonia have been the subject of numerous reports (1, 2). Sufficient data have accumulated to establish this disease as a definite clinical entity, distinct from the usual forms of bacterial pneumonia. Etiological studies, conducted in many laboratories, have yielded divergent results, although in some instances filtrable agents have been isolated (3). Laboratory investigations to date have been restricted considerably because of the lack of a uniformly susceptible animal host. At the present time, identity of the infectious agent, the mode of transmission, and nature of communicability of the disease must be considered unknown.

The purpose of this communication is to report the results of a preliminary experiment conducted in human volunteers inoculated with respiratory tract secretions of patients ill with primary atypical pneumonia. The principal objective of the study was to obtain data concern-

ing the transmissibility of the disease in the natural host.

METHODS AND MATERIALS

Site of experiment. The study was conducted at a Civilian Public Service camp near Gatlinburg, Tennessee, in the Great Smoky National Park. A group of buildings, formerly used by the Civilian Conservation Corps, provided facilities for accommodating approximately 140 men. A small infirmary, apart from the main dormitories and normally equipped to care for 9 patients, was used to house the volunteers.

Subjects. All of the men were conscientious objectors who had volunteered for the experiment. From a group of 23 men, 15 were selected, after careful clinical, bacteriological, and roentgenographic study, as suitable for inoculation. An additional group of 16 volunteers was chosen for the purpose of observing the patterns of respiratory illness at the camp during the conduct of the experiment.

Isolation. The facilities of the infirmary prevented strict isolation of each individual in the inoculated group. Twelve men were quartered in double-decked bunks in one large room, 2 men shared a room of moderate size, and 1 individual occupied a single room. The members of the entire group were in close contact with each other at all times. They shared a common lavatory. The men were allowed restricted out-of-door recreational privileges, but had no immediate personal contact with the other campers nor were they allowed to enter other buildings. The attending physicians and assistants wore gowns and masks when in contact with subjects in the infirmary.

The non-inoculated group of 16 men pursued their normal duties in or around the camp, lived in the common dormitories, shared the common mess hall, and were in normal contact with their fellows, but were not allowed furloughs.

Observation of non-inoculated group. This group of 16 men was observed 3 times weekly for evidences of respiratory disease, at which time a history of the presence and severity of certain constitutional and localizing respiratory symptoms was made. Significant physical signs were recorded. Roentgenograms of the chest were taken weekly throughout the experiment. Throat cultures were taken on each man prior to and during the study. A differential and total leukocyte count was also made at the beginning of the study. Sera were obtained from each man prior to and at the end of the experiment for the purpose of serological tests.

¹ This investigation was supported through the Commission on Acute Respiratory Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of The Surgeon General, United States Army, and by grants from the Commonwealth Fund, the W. K. Kellogg Foundation, the John and Mary R. Markle Foundation, and the International Health Division of the Rockefeller Foundation to the Board for the Investigation and Control of Influenza and Other Epidemic Diseases for the Commission on Acute Respiratory Diseases.

² Members of the Commission on Acute Respiratory Diseases are: J. H. Dingle, Major, M.C., A.U.S., Director; T. J. Abernethy, Major, M.C., A.U.S.; G. F. Badger, Captain, M.C., A.U.S.; J. W. Beard, M.D.; N. L. Cressy, Major, M.C., A.U.S.; A. E. Feller, M.D.; A. D. Langmuir, Captain, M.C., A.U.S.; C. H. Rammellkamp, M.D.; E. Strauss, Captain, M.C., A.U.S.; and Hugh Tatlock, 1st Lieutenant, M.C., A.U.S., National Research Council Fellow.

TABLE I

Clinical and laboratory data on patients from whom specimens were obtained

Patient	Lobes involved	Specimen		Sera		
		Day of disease	Type*	Day of disease obtained	Cold agglutinin titer	Strep. (Rockefeller No. 344) agglutinin titer
A. N.	L.L.	4	T.W.	5	8	10
				8	8	10
				16	—†	40
M. F.	L.L.	3	T.W. Sp.	3	16	20
				10	512	10
				18	—	20
W. K.	R.U., R.M., R.L., L.L.,	3	T.W.	3	0	20
				15	512	10
				20	256	20
L. R.	R.U., R.M., R.L.	3	T.W.	3	0	20
				17	65,536	40
				26	4,096	20
R. M.	R.L.	3	T.W.	3	—	20
				8	—	20
R. D.	R.L., L.L.	13	T.W.	12	256	20
				21	4,096	20
				32	256	20
R. S.	L.U., L.L., R.U., R.L.	12	T.W. Sp.	3	0	10
				12	16,384	40
				19	4,096+	160

* T.W. = Throat Washings; Sp. = Sputum.

† — = Test not done.

Inocula. Throat washings and sputa were collected from 7 patients admitted to the wards of the Regional Hospital, Fort Bragg, during the previous 4 months (Table I). Each of these patients was ill with the characteristic syndrome of primary atypical pneumonia (lg), confirmed by roentgenogram. Five of the 7 patients developed "cold agglutinins" in their sera during the course of the disease. Specimens were collected from 4 patients on the third day of the disease; in the remaining 3 cases, collections were made on the fourth, twelfth, and thirteenth days, respectively, during which time the patients were severely ill. Immediately after collection, the specimens were placed in lusteroid tubes and frozen in a carbon dioxide ice-alcohol bath. They were stored at -72° C. until ready for use.

Roentgenograms. The standard U. S. Army portable Picker field x-ray unit, equipped with gasoline-driven generator, was employed in taking all chest films. All exposures were made with the subject in the upright position and at a distance of 72 inches for $\frac{3}{4}$ second, using intensifying screens. Processing of all exposed films was accomplished at the camp by the use of the standard U. S. Army Developing Unit.

Cold agglutinins. Tests to determine the presence of cold agglutinins were performed on the sera of all patients, both in the inoculated and non-inoculated groups (4). A

modification of the technique originally advanced by Peterson, Ham, and Finland (5) was employed. The blood was allowed to clot at room temperature and the serum separated by centrifugation. Sera were then stored at 4° C. until ready for use. Serial 2-fold dilutions of serum in physiological salt solution were made in 0.5 ml. volume in Wassermann tubes; the final dilution in the first tube was 1:4. One half (0.5) ml. of 0.2 per cent washed Group 0 human cells (not over 24 hours old) was then added to each tube. The tubes were shaken vigorously and kept overnight at 4° C. Readings were made immediately upon removal of the tubes from the icebox. Only positive tests which failed to persist after the tubes had stood at room temperature for 1 hour were accepted. Titers of 1:32 or higher were considered significant.

Agglutination tests. The sera of all individuals, both in the inoculated and non-inoculated groups, were tested for the presence of agglutinins to an indifferent streptococcus. The strains employed were Rockefeller No. 344,³ as well as 19 individual strains isolated from throat washings and sputum obtained from both donors and recipients of the inoculum. The technique employed was that described by Thomas *et al.* (6).

³ This strain was kindly supplied by Dr. Frank L. Horsfall, Jr.

PRELIMINARY STUDIES

Epidemiology. Prior to the inoculation of volunteers, a brief epidemiological survey of the camp was made. The purpose of this survey was to determine the incidence of respiratory illnesses during the 12-month period preceding the initiation of the experiment, to secure evidence of the existence of primary atypical pneumonia at the camp, and to collect data regarding illnesses which had occurred among the men who volunteered for the experiment.

Tabulation of the monthly incidence of respiratory illnesses at the camp from October 1, 1942, to August 31, 1943, and during the time the experiment was in progress, showed the expected seasonal variations. The majority of these illnesses were short in duration and mild in nature, and simulated the common cold. Only a small proportion required infirmary care, and then usually for less than 1 week. No definite evidence could be found that atypical pneumonia had existed at the camp prior to the initiation of the experiment, although in some individuals the disease may have been present and unrecognized for lack of roentgenographic study. The illness in one patient may have been significant; he had had a chronic cough for several weeks and a single roentgenogram was interpreted as showing "pneumonitis and questionable bronchiectasis."

Roentgenographic survey. As an adjunct to the above study, a roentgenographic survey was undertaken at the camp immediately prior to the experiment. Single films of the chest were taken on 123 individuals who were either members of the camp or closely identified with its administration. In no instance was an infiltrative lesion characteristic of atypical pneumonia encountered.

EXPERIMENTAL STUDY

Prior to inoculation, the 15 volunteers were segregated in the camp infirmary. Over a period of 5 days (October 3 to 8, 1943), each volunteer was examined daily and careful note was made of the development of any respiratory symptoms. The oral temperature and the pulse rate were recorded at 4-hour intervals, daily; the respiratory rate was taken twice, daily. During this period, a throat culture, bleeding, complete blood count, urinalysis, and roentgenogram were performed on each patient. Twelve individuals experienced no respiratory symptoms during this period of preliminary observation; 3 individuals developed respiratory illnesses as follows:

T. W. on October 4 noted headache, sneezing, slight soreness of nasopharynx, hoarseness, and dry cough. Physical examination was essentially negative. On October 6, coryza, nasal obstruction, and discharge were more evident. Symptoms of a mild respiratory illness without fever or significant physical signs continued for approximately 10 days. He was not inoculated but was not removed from the experimental group.

D. M. developed symptoms and signs of a mild afebrile respiratory infection 36 hours after entering the infirmary. The principal symptoms were those of coryza. No significant physical signs were apparent. By October 10, he

was considerably improved and received 1 day's inoculations.

V. M. noted sneezing, dryness of the nasopharynx, and sore throat on October 6, 3 days after entry into the infirmary. Symptoms and signs of a mild infection of the upper nasal passages continued for 1 week. The patient remained afebrile throughout. He was not inoculated but was kept in the experimental group.

The illnesses of these 3 individuals complicated the experiment considerably. These infections were mild in nature, however, and similar in their manifestations to infections occurring simultaneously in other campers. None of the 3 men developed roentgenographic evidence of pulmonary infiltration at this time or subsequently.

Notwithstanding the development of these minor illnesses, the inoculations were given to the other 12 volunteers over a period of 3 days (October 8 to 10). Each patient received an inoculation of unfiltered throat washings (in meat-infusion broth or Ringer's solution) as well as a pool of unfiltered throat washings and sputa intranasally and by inhalation, 3 times daily, for 3 consecutive days. The intranasal inoculations were given with the aid of a standard nasal atomizer. The inhalation of inoculum was accomplished by the use of a nebulizer⁴ which emitted a fine cloud of vapor. Patients were instructed to inhale deeply 50 times as the mouth of the nebulizer was held below the soft palate. With each inhalation, the fine vapor of inoculum could be seen being sucked downward toward the lower respiratory tract. Immediately following the separate inoculations, patients reclined in the supine position with the head extended slightly, for a period of 15 minutes. Each of the 12 patients received the full course of inoculations in an identical manner, and approximately 8 ml. were given to each man. One additional volunteer (D. M.) was given 1 day's inoculation, totaling 2.5 ml.

During the post-inoculation period of 5 weeks, the volunteers were observed daily for the development of symptoms and physical signs of respiratory disease. Observations of the temperature, and of the pulse and respiratory rates, were continued. Roentgenograms of the chest were taken twice weekly, or oftener if indicated. Leukocyte counts with Schilling differential indices were performed twice weekly. Blood for serological study was obtained at weekly intervals. Throat cultures were taken weekly. Patients in need of complete bed rest and nursing care were transported to a nearby hospital.

RESULTS IN NON-INOCULATED GROUP

Clinical findings. Respiratory illnesses of mild severity and short duration developed in 13 of the 16 individuals who were permitted to pursue their normal duties about the camp but who received no inoculation. The time of development of respiratory disease in this group is shown in Figure 1. Three patients pre-

⁴ Made by Vaponefrin Company.

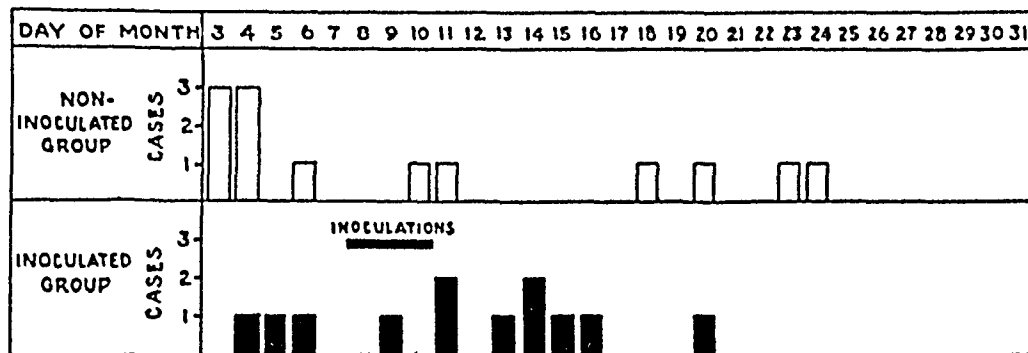


FIG. 1. DAY OF ONSET OF RESPIRATORY DISEASE IN INOCULATED AND NON-INOCULATED GROUPS

Note: Three patients in non-inoculated group presenting evidence of respiratory infection on October 3 developed symptoms on September 24, October 1 and 3, respectively.

sented evidence of mild upper respiratory infections when first examined on October 3. These illnesses began on September 24, October 1, and 3, respectively. Three additional patients developed symptoms on October 4. During the succeeding 5 weeks, respiratory disease developed in the remainder of the group in sporadic fashion. Constitutional symptoms (chilliness, malaise, or headache) during the course of their infection were reported by only 3 individuals (Figure 2). Localizing symptoms, *e.g.*, coryza, sore throat, and occasionally cough and hoarseness, were the predominant features of these illnesses (Figure 3). Roentgenograms of the lungs were consistently negative in 15 individuals. In only 1 case was there suggestive evidence of pulmonary infiltration accompanying the respiratory infection. The clinical history on this patient's illness follows:

On October 3, K. B. inhaled a considerable amount of smoke while engaged in fighting a forest fire. That evening he noted a slight sore throat. The following day he complained of headache, sneezing, coryza, hoarseness, and cough. He reported to the dispensary, where his temperature was found to be 99.4° F. and he was placed in quarters. On October 5, his temperature was normal but the symptoms continued. On October 6, his symptoms were unchanged and the physical examination revealed tender lymph nodes, nasal obstruction and discharge, a small patch of exudate on the pharynx, and scattered rhonchi over the lungs. Roentgenograms of the lungs taken on September 29, before the development of this infection, and on October 6, showed no pulmonary lesion. By October 8, he was sufficiently improved to be discharged from the infirmary, but cough and nasal symptoms continued. Physical examination at this time disclosed only tender cervical lymph nodes; no pharyngeal exudate was visible and the lungs were clear. During the next 10 days, while on duty, he was aware of intermittent headache, malaise, nasal discharge, sore throat, and cough, but nothing of significance was found on physical examination. A roentgenogram of the chest on October 14

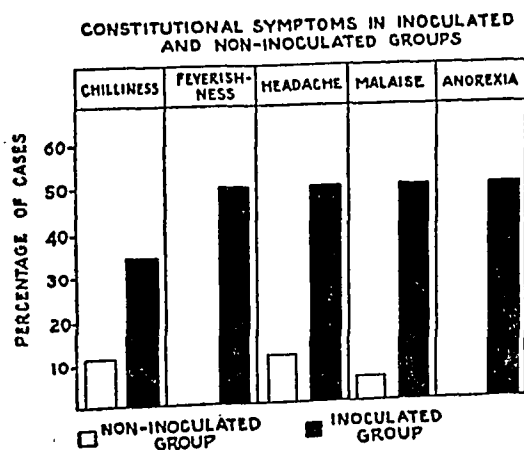


FIG. 2. CONSTITUTIONAL SYMPTOMS IN INOCULATED AND NON-INOCULATED GROUPS

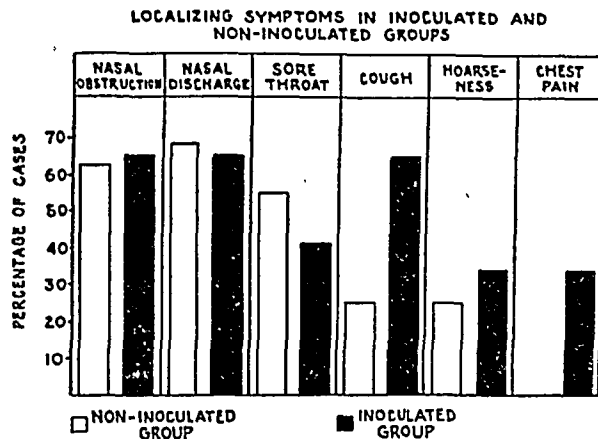


FIG. 3. LOCALIZING SYMPTOMS IN INOCULATED AND NON-INOCULATED GROUPS

was again negative. He was readmitted to the infirmary on October 18 because of exacerbation of all symptoms the previous night. He complained of cough and a dull frontal headache, and there was tenderness on pressure over the frontal sinuses, together with a purulent post-nasal discharge. His temperature remained normal. On October 21, a roentgenogram of the chest was taken, which has since been interpreted as showing a questionable area of increased density of the mesial portion of the left lung. Opinion of the roentgenologists is divided as to whether this lesion represents peribronchial pneumonia or increased bronchovascular markings. The patient was able to resume his duties on October 27. A roentgenogram of the chest on November 2 was negative. No fine râles over the chest were detected at any time. The clinical diagnosis was sinusitis, pharyngitis, and bronchitis. One specimen of serum (November 5) was tested for the presence of cold agglutinins but none was detected.

Laboratory findings. Throat cultures from the non-inoculated group, prior to and during the course of the experiment, showed no significant deviation from the normal flora. One individual with a mild respiratory infection was shown to have a moderate growth of β -hemolytic streptococcus on a single culture. There was no evidence that this organism had spread to other individuals in the group. Leukocyte and differential counts on this group were normal.

Tests for the presence of cold agglutinins and agglutinins for an indifferent streptococcus (Rockefeller No. 344) were performed on speci-

mens of sera taken prior to the experiment and 1 month later. Cold agglutinins were not present in significant titer (1:32) and there was no rise in titer of agglutinins for the streptococcus.

RESULTS IN INOCULATED GROUP

Respiratory illnesses developed in 10 of the 12 volunteers receiving the full 3 days' course of inoculations. In general, these illnesses were more severe and differed sharply from the infections observed in the non-inoculated group. The important data obtained following inoculation are given in Table II and in summary form below.

Day of onset of respiratory disease. Figure 1 shows the time of appearance of any symptom marking the onset of respiratory disease. In 3 individuals, respiratory disease began either during or immediately following the inoculations and was manifested by symptoms and/or signs localized to the nose or throat. Five individuals noted respiratory symptoms beginning from 5 to 8 days (October 13 to 16) following the first inoculation; one man noted symptoms on the twelfth day (October 20) and one noted symptoms on the twenty-second day (October 30). The development of the majority of these illnesses over a period of approximately 1 week

TABLE II
Clinical and laboratory data on patients in experimental group

Case No.	Patient	Age	History												Phys. exam.	Fever >99° F.			N-ray	Laboratory	
			Constitutional symptoms					Localizing symptoms								Dates	Duration (days)	Maximum		Cold agglutinins	Strept. No. 344 agglutinins
			Chills	Feverishness	Headache	Malaise	Anorexia	Nasal obstruction	Nasal discharge	Sore throat	Hoarseness	Cough	Sputum	Chest pain	Fine rales						
1	W. A.	19	+	+	+	+	+	+	+	+	+	+	+	+	+	10/17-10/23	7	101.6	+	+	
2	E. B.	19	+	+	+	+	+	+	+	+	+	+	+	+	+	10/13-10/14	2	99.6	+	+	
3	J. B.	25	+	+	+	+	+	+	+	+	+	+	+	+	+	10/17-10/27	11	101.8	+	+	
4	W. H.	25	+	+	+	+	+	+	+	+	+	+	+	+	+	11/2, 11/4	2	99.8	+	+	
5	R. K.	21	+	+	+	+	+	+	+	+	+	+	+	+	+	10/23-10/25	3	100.2	+	+	
6	V. M.	24	+	+	+	+	+	+	+	+	+	+	+	+	+	10/23, 10/25	2	99.6	+	+	
7	L. M.	24	+	+	+	+	+	+	+	+	+	+	+	+	+	10/15-10/22	7	102.0	+	+	
8	D. M.	18	+	+	+	+	+	+	+	+	+	+	+	+	+	10/30, 11/2	4	102.0	+	+	
9	K. M.	23	+	+	+	+	+	+	+	+	+	+	+	+	+				+	+	
10	E. O.	23	+	+	+	+	+	+	+	+	+	+	+	+	+				+	+	
11	D. P.	25	+	+	+	+	+	+	+	+	+	+	+	+	+				+	+	
12	O. S.	25	+	+	+	+	+	+	+	+	+	+	+	+	+				+	+	
13	D. T.	25	+	+	+	+	+	+	+	+	+	+	+	+	+				+	+	
14	T. W.	25	+	+	+	+	+	+	+	+	+	+	+	+	+				+	+	

* Not inoculated.

** Received 1 day's inoculation Oct. 10, 1943.

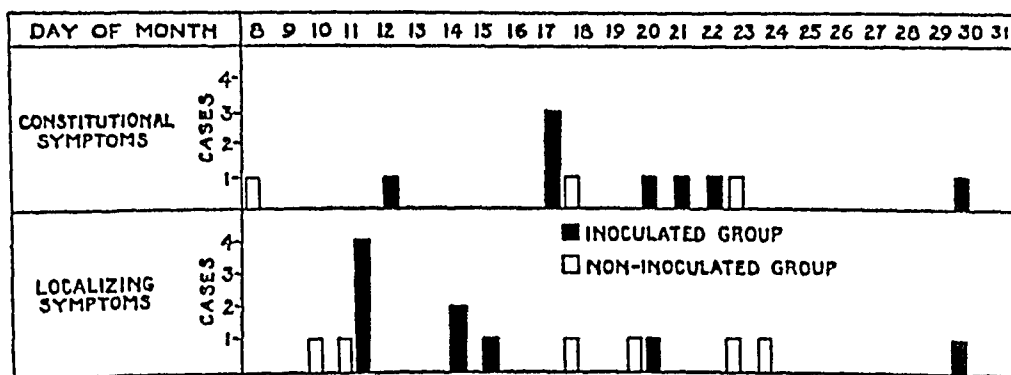


FIG. 4. DAY OF ONSET OF ANY CONSTITUTIONAL OR LOCALIZING SYMPTOM FROM DATE OF INOCULATION IN INOCULATED AND NON-INOCULATED GROUPS

following the first inoculation is in sharp contrast to that observed in the non-inoculated group.

Constitutional symptoms. Seven patients developed significant constitutional symptoms which were generally of moderate severity, although in 3 patients (W. A., R. K., and O. S.), some of the symptoms were present in severe form (Table II). One individual (L. M.) presented only a single constitutional symptom. Chilliness was noted by 4 patients (Figure 2). In no instance was a frank rigor observed. Subjective sensation of feverishness was present in 6 patients. Dull frontal headache, malaise, and anorexia were each present in 6 cases. The time of development of constitutional symptoms differed somewhat among the 12 inoculated individuals (Figure 4). Three men noted onset of symptoms on October 17, 9 days following the first inoculation.

Localizing symptoms. Symptoms, localized either in the upper or lower respiratory tract, were present in 9 patients (Table II, Figure 3). Nasal obstruction and discharge were present in each of 8 patients. Sore throat, usually of mild severity, was noted by 5 individuals. Hoarseness was noted in one-third of the patients. Cough, usually of a dry paroxysmal character in the early stages, was observed in 8 patients. In 2 of these, the cough was severe, and in 7, it became productive. The sputa were invariably mucoid or mucopurulent and in no instance contained fresh or altered blood. Chest discomfort consisting of substernal "soreness" or "rawness" was observed by one-third of the patients. None of the patients developed a pleural type of

pain. The day of onset of these localizing symptoms is shown in Figure 4.

Physical examination. Characteristic "sticky" subcrepitant râles (lg) were detected in the lungs of 5 patients. In 2, the râles were persistent and continuously heard over localized areas. In the remaining 3 patients, the râles were localized to given areas but were transitory in nature. Rhonchi were audible in 4 patients. Abnormal signs in the nose and throat were detected in 8 patients having symptoms from these regions; these signs, however, were usually minimal. Two patients developed purulent discharge from the nose. Moderate injection of the throat usually accompanied symptoms of sore throat. The remainder of the physical examination showed no abnormalities.

Fever. Eight patients developed fever (99° F. or above) following the inoculations (Table II). The temperature charts of these patients (Figure 5) show variability not only in the time of development (October 13 to November 4) but also in the height of the temperature response (99.2 to 102° F.). There were 5 individuals (Cases 11, 1, 5, 8, and 14) who showed maximum temperatures of 100° F. or above and who had associated symptoms of respiratory illness. The development of significant fever was first observed in these individuals in a period varying between 7 and 22 days after the first inoculation. Three of these 5 men showed febrile responses beginning 7 to 9 days after the first inoculation. There were 3 volunteers who developed slightly abnormal temperatures of 1 to 2 days' duration (Cases 1, 9, and 6). Only 1 individual (Case 3)

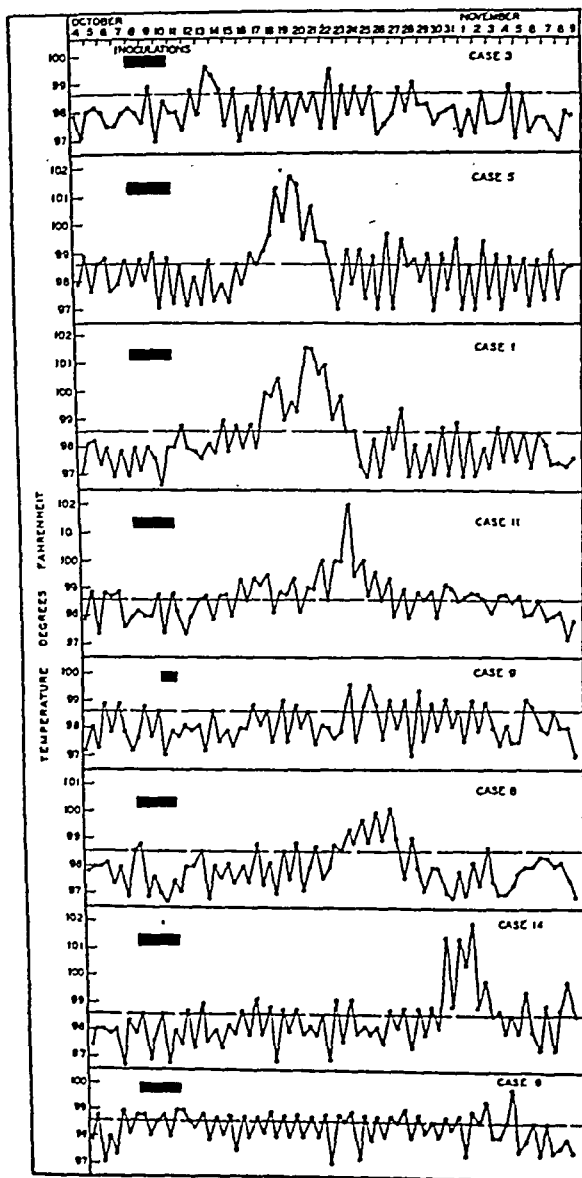


FIG. 5. COMPOSITE TEMPERATURE CHARTS OF 8 VOLUNTEERS WHO DEVELOPED FEVER (99° F. OR ABOVE) FOLLOWING THE INOCULATIONS

in the latter group manifested systemic symptoms of illness.

Röntgenograms. On review of all films without knowledge of the clinical history, 1 patient (R. K., Case 5), in the opinion of the roentgenologists, showed evidence of a patchy infiltration of both lung bases, more marked on the left. This patient had the characteristic symptomatology and physical signs usually accompanying primary atypical pneumonia. The films of 2 other pa-

tients (W. A., Case 1, and L. M., Case 8) showed similar findings in single lobes, but to a less marked degree. Patients R. K. and W. A. were sufficiently ill to be hospitalized. In these instances, however, the pulmonary infiltrations were mild in degree and not so extensive as those usually observed in atypical pneumonia as seen in a hospital ward.

Laboratory findings. Cold agglutinins were detected in the sera of 3 patients (Table II). One patient (L. M., Case 8), who developed a febrile illness of 6 days' duration with accompanying symptoms and signs of respiratory disease, had a cold agglutinin titer of 1:128 on October 28 and 1:256 on October 31. Another patient (P. O., Case 11) developed cold agglutinins in titer of 1:64 following a febrile illness of 12 days' duration. A third patient (W. A., Case 1) showed a cold agglutinin titer of 1:128. Tests on the sera of the remaining patients were negative.

Agglutination tests with 20 strains of indifferent streptococci (Rockefeller No. 344, and 19 additional strains) showed no definite rises in antibody titers in the sera of any of the patients receiving inoculations.

Tests for the development of antibodies against influenza viruses A (PR8) and B (Lee) were done on samples of sera collected before and 3 weeks following the inoculations, employing the chicken red blood cell agglutination inhibition test of Hirst (7). No evidence of an increase in antibody titers to either virus was demonstrable in any of the specimens of convalescent serum.

With two exceptions, the leukocyte and Schilling differential counts in these patients showed no significant deviation from normal throughout the experimental period, despite the development of fever and/or severe respiratory symptoms. Two patients who were severely ill and required hospitalization developed a leukocytosis of 12,000 with an absolute increase in the total number of polymorphonuclear neutrophils during the febrile period.

Results of weekly throat cultures showed no significant changes in bacterial flora immediately following the inoculations. The types of organisms present were those usually encountered in routine cultures from normal individuals with-

out respiratory disease. α -streptococci, *H. influenzae*, and gram-negative cocci (not further identified) were consistently predominant organisms throughout the experimental period. Beginning on October 20, however, β -hemolytic streptococci were recovered from 1 patient. On October 26 and again on October 29, similar organisms in moderate numbers were isolated from 4 other cases. On November 3, only 1 patient was found to be still harboring these organisms. There was no evidence that the presence of β -hemolytic streptococci was associated with a clinical illness in any of these patients.

Case Reports

Case 5, Figure 6. R. K., aged 21, had enjoyed good general health immediately prior to the experiment. His past history was marked by a severe attack of tonsillitis in 1936. He usually experienced one or two "head colds" a year, lasting approximately 7 days. The most recent infection occurred in February 1943. During the pre-inoculation period, no significant respiratory symptoms or signs were observed.

Mild nasal obstruction and discharge developed concurrently with the inoculations. On October 11, a dry cough developed which increased in severity so that by October 15 it was keeping the patient awake at night.

On October 17, the patient complained of chilliness, dull supra-orbital headache, and substernal discomfort following each cough. He did not appear ill at this time and the physical examination was essentially negative. The following day the patient appeared definitely ill for the first time. Constitutional symptoms of chilliness, feverishness, headache, malaise, and anorexia were conspicuously present and the temperature rose to 101.6° F. Physical examination disclosed only questionable dullness over the right hilus posteriorly. On October 19, the patient's fever continued and all symptoms became aggravated. Examination revealed numerous fine and coarse moist râles at both bases, without other signs of infiltration. On this date, he was transferred to a hospital for further care, where he stayed for approximately 3 weeks. A roentgenogram of the chest on October 20 showed evidence of a bilateral infiltrative lesion consistent with that of primary atypical pneumonia. These findings were observed on successive daily films taken over the course of the next week, but with gradual clearing of the pneumonic process during this time. The febrile course was of 11 days' duration. Constitutional symptoms persisted until November 2. Fine râles were continuously heard over a period of approximately 1 week. Except for a cough lasting until November 7, the patient made an uneventful recovery.

Comment. An acute febrile illness of moderate severity developed 9 days after the first inoculation. Symptoms and signs suggestive of

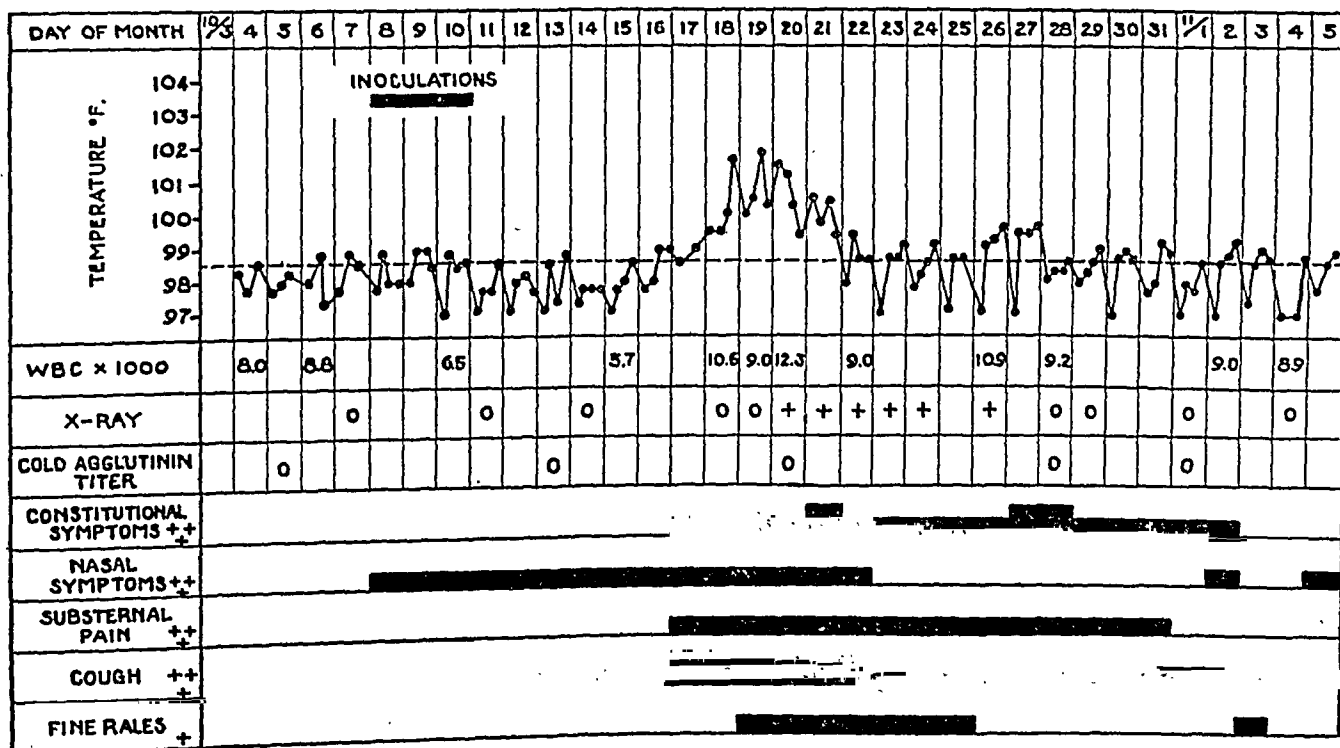


FIG. 6, CASE 5. CLINICAL CHART OF PATIENT WHO DEVELOPED AN ACUTE FEBRILE RESPIRATORY ILLNESS RESEMBLING PRIMARY ATYPICAL PNEUMONIA

Note: + = symptom or sign present and of mild degree; ++ = symptom or sign present and of moderate severity.

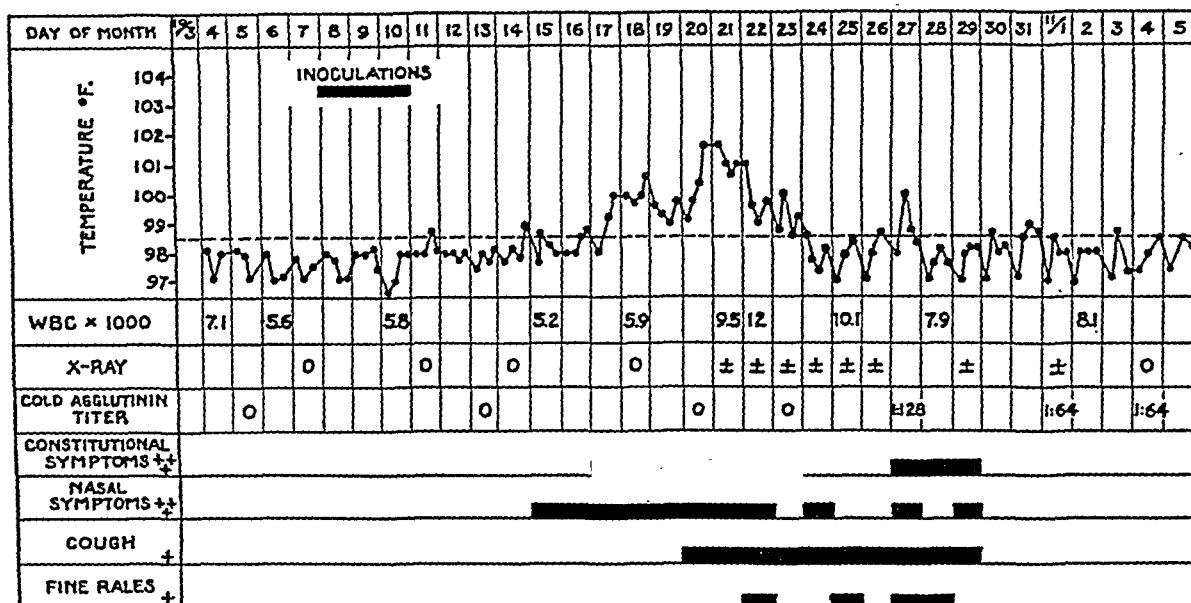


FIG. 7, CASE 1. CLINICAL CHART OF PATIENT WHO DEVELOPED AN ACUTE FEBRILE RESPIRATORY ILLNESS RESEMBLING PRIMARY ATYPICAL PNEUMONIA

For explanation of symbols, see Fig. 6.

primary atypical pneumonia were noted. Evidence of a bilateral infiltrative lesion was obtained by roentgenograms of the chest. Convalescence was prolonged but the patient made an uneventful recovery.

Case 1, Figure 7. W. A., a 19-year-old camper, had always enjoyed good health except for minor head colds of short duration, occurring 3 or 4 times a year, and for a "streptococcus throat infection" in 1938, following which tonsillectomy was performed. Examination prior to the experiment disclosed no abnormal respiratory symptoms or signs except for marked deviation of the nasal septum.

No symptoms developed during the course of the inoculations. On October 14, the patient complained of slight sore throat and hoarseness; however, the physical examination was negative. On October 15, coryza and nasal obstruction were noted. Two days later, constitutional symptoms developed, consisting principally of headache, chilliness, malaise, anorexia, and fatigability. The temperature, which had previously been normal, rose to 100° F. The patient looked mildly ill. All symptoms became aggravated the following day, and fever varied between 99.8° and 100.6° F. His condition was somewhat improved on October 19 and the temperature was lower. Constitutional symptoms continued, and he was still mildly ill. Physical examination disclosed no significant abnormalities of the respiratory tract. With the onset of cough and severe constitutional symptoms on October 20, the patient appeared more ill. There was questionable dullness to the percussion note at the right base, but no

râles. He was transferred to a hospital on October 21. On this date, the lungs were negative to physical examination, but the roentgenogram revealed exaggerated markings in the mid-lung field adjacent to the right heart border. This abnormality gradually cleared over the next 10 days. Opinion of roentgenologists was divided as to the significance of the changes observed. Examination of the chest at daily intervals failed to reveal fine "sticky" râles over the area shown roentgenographically to be abnormal. A few fine râles, however, were audible at intervals over the left hilus. The temperature fell to normal by lysis on October 24 and thereafter remained normal. Convalescence was slow and there were no complications. Tests for the development of cold agglutinins in the serum were done subsequent to recovery. These showed a titer of 1:128 on October 27, with a progressive fall in titer in the samples taken on November 1, 4, and 12.

Comment. The illness in this patient was of moderate severity. Constitutional symptoms, which marked the onset of the acute febrile course, developed 9 days after the first inoculation. The symptoms were characteristic of those usually associated with atypical pneumonia. Neither physical signs nor definite roentgenographic evidence of the disease, however, developed with any degree of certainty. The course of the disease was consistent with that of a moderately severe case of atypical pneumonia.

Case 11, Figure 8. P. O., aged 19, gave a history of repeated infections of the respiratory tract during the past few years. These consisted principally of a head cold and sore throat which occurred approximately 6 times a year, lasting less than a week at a time. He had pneumonia in 1931 and 1936, and sinusitis and mastoiditis in 1934. During the previous 3 months, he had been free of respiratory infections.

The patient remained well during and immediately following the inoculations. Beginning on October 15 and continuing for approximately a week, there was slight afternoon or evening elevation of temperature to 99.4° or 99.6° F. There were no accompanying complaints and the patient was normally active. On the 21st and 22nd of October, the temperature rose to 100° F. At this time, he noted feverishness, chilliness, and frontal headache, but no abnormal physical findings were observed. He became mildly ill on October 23 with the development of fever (102° F.), chilliness, feverishness, malaise, anorexia, sneezing, and slight nasal discharge. Except for moderate injection and hypertrophy of the pharyngeal lymph follicles, no significant physical signs were noted. The patient stated that his symptoms were more severe than those usually experienced with respiratory infections. On October 24, the temperature was lower and constitutional symptoms had abated materially. The abnormalities previously noted in the throat were still present. The lungs were clear by auscultation. The temperature fell by lysis and there was corresponding symptomatic improvement. On only one occasion, October 31, were a few fine râles detected over the right axilla which persisted after coughing. Roentgenograms taken repeatedly, during and subsequent to the febrile course, failed to reveal evidence of pulmonary infiltration at any time. The patient made an uneventful convalescence.

Tests for the development of cold agglutinins were performed on the patient's sera, collected at weekly intervals throughout the period of observation. Agglutinins in low but significant titer (1 : 64) were detected in the serum sample of October 28.

Comment. This patient's illness consisted of an initial asymptomatic period, characterized by low-grade fever beginning 7 days after the first inoculation, which was followed by a sharp febrile symptomatic period of 1 week's duration. The symptoms were of moderate severity. Râles in the lungs were heard on only one occasion, late in the course of the disease. The roentgenograms did not show abnormal pulmonary shadows. Sera of the patient revealed the presence of cold agglutinins in significant titer. Except for the negative roentgenograms and the paucity of confirmatory lung signs, the patient developed a mild respiratory infection consistent with the diagnosis of atypical pneumonia.

Case 8, Figure 9. L. M., 24 years old, had always enjoyed good health. He usually experienced 2 head colds each winter, which lasted 2 or 3 days and were uncomplicated. His most recent respiratory infection occurred in January 1943.

No significant symptoms or signs developed during the pre-inoculation period or immediately following the inoculations. The patient was well until 12 days after the first inoculation, when he noted dryness of nose and pharynx, hoarseness, and a "tickling" cough, symptoms

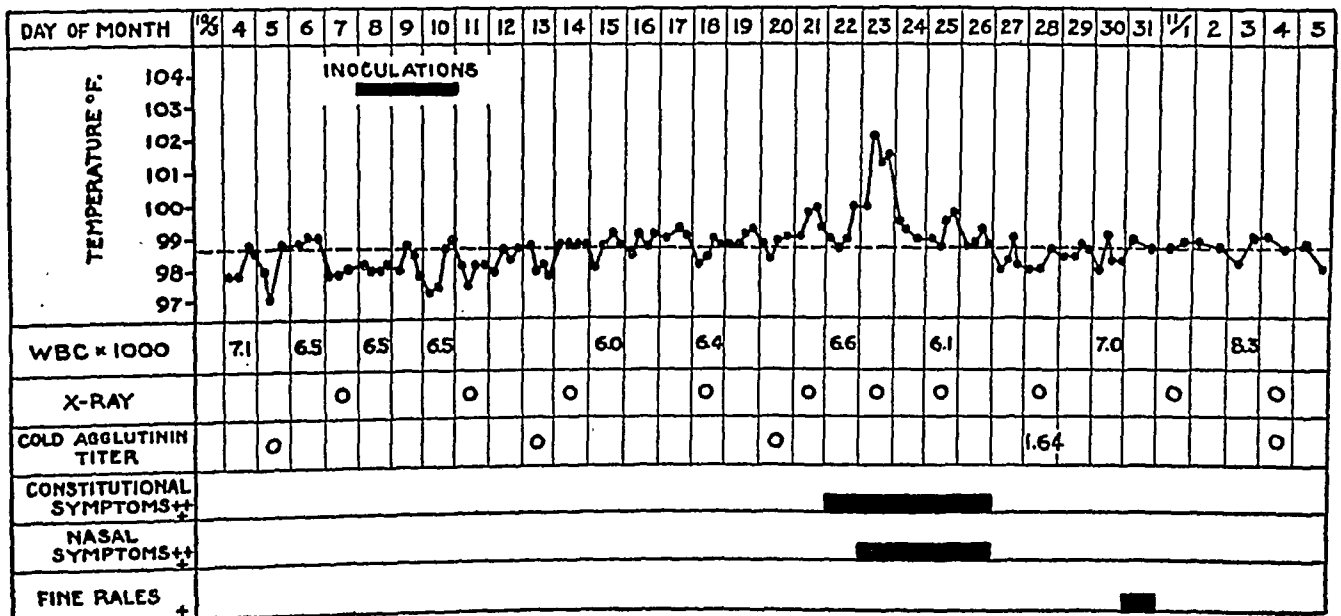


FIG. 8, CASE 11. CLINICAL CHART OF PATIENT WHO DEVELOPED A MILD FEBRILE RESPIRATORY ILLNESS FOLLOWING INOCULATION

For explanation of symbols, see Fig. 6.

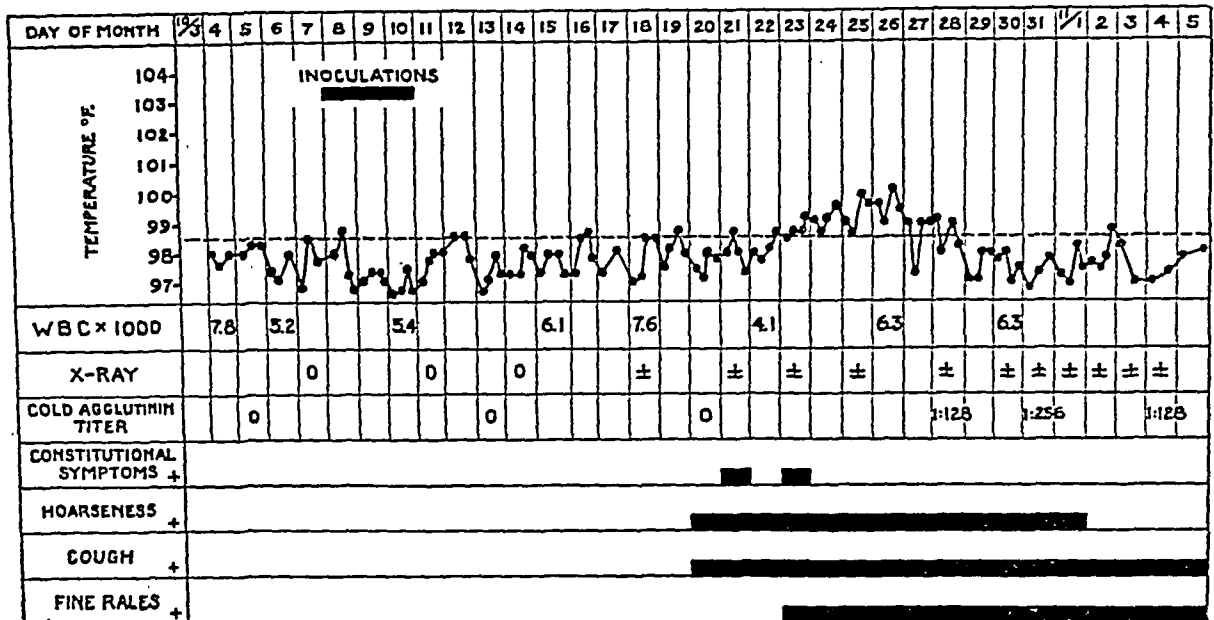


FIG. 9, CASE 8. CLINICAL CHART OF PATIENT WHO DEVELOPED A MILD RESPIRATORY ILLNESS RESEMBLING PRIMARY ATYPICAL PNEUMONIA

For explanation of symbols, see Fig. 6.

which were unlike those usually experienced with a cold. On October 21, there was slight nasal obstruction, injection of the pharynx, exudate on a portion of the pharyngeal lymphoid tissue, and aggravation of the cough. His symptoms continued, with dry cough and hoarseness as the most prominent features. On October 23, he noted slight feverishness, and fine inspiratory râles and rhonchi were detected for the first time over the lung bases. Slight evening elevation of temperature was noted on this date, and a low-grade fever continued during the next 5 days. The patient was less active than usual but not sufficiently ill to require bed rest. Râles and rhonchi persisted and were heard daily over both lung bases and over the right middle lobe. Rhonchi were no longer heard after November 2; a few râles were audible until November 11, a period of approximately 3 weeks. Cough persisted for about 4 weeks and eventually cleared. There were no complications.

Roentgenograms of the chest were consistently negative until October 18. On this date, the film showed increased bronchovascular markings at the left base. One roentgenologist who reviewed the films believed the infiltration was of sufficient degree to represent definite peribronchial pneumonia. The films taken on October 21 and 23 showed no change. On October 25, the increased markings were less evident. Residual changes were visible in frequent films taken over the course of the next 2 weeks. A final film on November 7 still showed a residual amount of increased markings at the left base.

Cold agglutinins developed in the sera of this patient with the cessation of the febrile period. A sample taken on October 28 gave a titer in dilution of 1:128. Subse-

quent samples taken on October 31 and November 4 showed titers of 1:256 and 1:128, respectively.

Comment. This patient's illness was marked by an insidious onset, minimal constitutional symptoms, a short febrile course, protracted cough and lung signs, and questionable roentgenographic evidence of pulmonary infiltration. The illness was extremely mild and not incapacitating. Cough and hoarseness were the predominant symptoms. The development of cold agglutinins, together with the auscultatory findings, suggested the diagnosis of mild atypical pneumonia.

DISCUSSION

In this study, an attempt has been made to transmit primary atypical pneumonia in human volunteers. At the beginning of the experiment, it seemed of importance to determine whether or not atypical pneumonia existed at the camp, either in the immediate or in the remote past. For this purpose, a survey was conducted which showed a normal seasonal prevalence of respiratory disease in the 12-month period preceding the initiation of the experiment. The data available from the dispensary records indi-

cated that definite cases of pneumonia probably had not occurred. In addition, a roentgenographic survey, consisting of single films in 123 individuals, revealed no instance of atypical pneumonia. There was thus an indication that the disease under investigation was not then present in recognizable endemic form at the camp and that infections, if they could be produced successfully, might reasonably have resulted from the inoculations.

The observations made in this preliminary experiment indicate that respiratory disease of variable clinical patterns developed in a considerable proportion of human volunteers following inoculation with unfiltered secretions obtained from the respiratory tracts of patients with primary atypical pneumonia. The clinical course of illness in 3 individuals (Cases 1, 5, and 8) was similar to, if not identical with, that usually observed in patients with primary atypical pneumonia (lg). These 3 individuals showed suggestive roentgenographic evidence of pneumonia and 2 patients were sufficiently ill to be hospitalized. In another individual (Case 11), an illness resulted which was indistinguishable from atypical pneumonia except for the absence of roentgenographic evidence of the disease. This type of illness has previously been termed "bronchitis resembling atypical pneumonia" (lg). Six additional patients (Cases 2, 3, 4, 12, 13, and 14, Table II) developed mild acute illnesses of the respiratory tract without pulmonary lesions demonstrable either by physical examination or by roentgenogram. Thus, there is a limited amount of experimental evidence from this preliminary study to indicate a possible relationship between atypical pneumonia and certain mild illnesses of the respiratory tract without accompanying pulmonary lesions. Others (8, 9a and b) have previously noted this relationship. Clinical and epidemiological evidence obtained from the study of atypical pneumonia at Camp Claiborne, La. (lg), and more recently at Fort Bragg (10a and b), also has supported this concept.

The course of illness which developed in these volunteers bore little resemblance to that usually observed either in individuals with the "common cold" or in subjects inoculated with filtered nasal secretions from patients with early acute

"colds." Although symptoms and signs of inflammation of the nose and throat were noted in approximately two-thirds of the volunteers, it seemed significant that these manifestations were mild and often accompanied by definite constitutional symptoms and fever. Furthermore, two other groups of workers (11, 12) have shown that in human transmission experiments the incubation period of colds is generally short, usually 24 hours or less and probably not exceeding 4 days. Evidence from this experimental study indicates an incubation period of the acute illness in excess of 4 days. In view of the fact that unfiltered inoculum was used, however, and that mild respiratory disease was present in 3 volunteers at the beginning of the experiment, the possibilities exist that these infections may represent either bacterial complications of the "common cold" or instances of undifferentiated respiratory disease. These possibilities cannot be excluded with certainty, although the clinical course of disease in the inoculated patients did not support either concept.

The nature of illness developing in these volunteers likewise bore little resemblance to the classical picture of influenza, either experimentally or naturally acquired (13). Two other investigators (14, 15) have shown that an acute illness of abrupt onset usually follows within 24 to 48 hours after the experimental inoculation of influenza virus A into human volunteers. The subjects in this study developed their illnesses more than 48 hours after inoculation, and neither the clinical characteristics nor course of disease resembled typical influenza. Furthermore, none of the patients nor the men in the non-inoculated group showed serological evidence of an antibody rise to influenza viruses A or B during the period of the experiment.

The lack of more definite evidence of pulmonary infiltration in any of the roentgenograms was an unexpected finding, in consideration of the severity of illness manifested by some patients. The soft, patchy lesion with greatest density near the hilus, which is observed so frequently in atypical pneumonia, was not encountered. The maximum changes observed consisted of greatly exaggerated bronchovascular markings, which were seen to develop gradually at the base of one or more lobes and to undergo

slow resolution over a period varying from 1 to 2 weeks. These findings, although minimal, were considered significant, and may be of importance to the concept of the possible relationship of certain forms of bronchitis to atypical pneumonia (lg).

In view of the serological reactions with an indifferent streptococcus (Rockefeller No. 344) obtained in certain cases of atypical pneumonia (6), a study was made of the relationship of this bacterium and other strains of indifferent streptococci to the respiratory illnesses occurring not only in the donors, but also in the recipients of the inoculum, *i.e.*, the volunteers. Nine strains of indifferent streptococci were isolated from the inocula used in the experiments. None of these showed specific agglutination by anti-344 rabbit serum, by the convalescent sera of the donors, nor by a known positive convalescent human serum. Three of the donors, however, did show a rise in agglutinin titer to streptococcus No. 344 in samples of convalescent serum (Table I). From the volunteers, 26 additional strains of indifferent streptococci were isolated. Nineteen of these when tested showed non-specific agglutination reactions with rabbit immune and human convalescent serum. All samples of serum obtained from the 15 volunteers and the 16 men in the non-inoculated group were tested against No. 344 and the 19 strains isolated from the recipients. None of these samples of serum showed a rise in agglutinin titer to any of the strains. This evidence indicates that the indifferent streptococcus probably did not play a significant etiological rôle in the illnesses developing in any of the volunteers.

It has been suggested (5) that "the development of cold agglutinins may serve as a criterion for segregating some of the prevalent cases of primary atypical pneumonia until definite etiological agents are established." The inocula used in these experiments were obtained from 7 patients with primary atypical pneumonia, 5 of whom were shown to possess high titers of cold agglutinins in their sera. Three of the volunteers who received the inocula developed significant titers of cold agglutinins approximately 1 week following the onset of the febrile period. It has been found in this laboratory (4) that cold agglutinins first appear in the sera of some pa-

tients with atypical pneumonia at approximately this time. Furthermore, the incidence of positive reactors in this study (25 per cent) compares favorably with what has been found in patients at Fort Bragg. Approximately 35 per cent of patients with atypical pneumonia may show the phenomenon at some stage of illness. It seems probable from the foregoing observations, therefore, that the clinical illnesses in the 3 inoculated volunteers who developed cold agglutinins were closely related to those in the patients originally studied at Fort Bragg and from whom the inocula were obtained.

SUMMARY

A preliminary experiment has been conducted in human volunteers in an attempt to transmit primary atypical pneumonia. Unfiltered throat washings and sputa obtained from patients early in the course of the characteristic disease were inoculated into the respiratory tract of 12 individuals. The volunteers were kept in group-isolation for a period of 6 weeks and observed daily for the development of any signs of respiratory illness.

Respiratory illnesses developed in 10 of the 12 volunteers. These illnesses varied considerably not only in severity, but also in clinical manifestations, one from the other. They differed sharply from infections observed in a similar group of non-inoculated individuals.

Evidence is presented to indicate that illnesses closely resembling primary atypical pneumonia developed in certain of those who received the inoculations.

The members of the Commission wish to express their gratitude to the administrative staffs of Selective Service, the National Service Board for Religious Objectors, and the American Friends Service Committee for the cooperation which has made these studies possible. Mr. E. Gordon Alderfer of N.S.B.R.O. materially assisted this investigation from its inception. Dr. Alex M. Burgess, Jr., Medical Director, American Friends Service Committee, gave his complete support to the study. Without the continued enthusiastic cooperation of Dr. John H. Ferguson, Director of Civilian Public Service Camp No. 108, and his administrative and medical staffs, this investigation would have been considerably restricted. Special thanks are due Major J. Ross Eakin, Superintendent of the Great Smoky National Park, for supplying the forestry project-work of the volunteers during the conduct of the experiment.

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THE EFFECTS OF TESTOSTERONE AND OF TESTOSTERONE PROPIONATE ON RENAL FUNCTIONS IN MAN

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The renotropic effects of testosterone and testosterone propionate have been demonstrated repeatedly in mice and rats (1, 2, 3). Whether or not the induced increase in size and weight of the organ is associated with any enhanced functional capacity thus far has not been determined. It has been found, however, that the administration of testosterone propionate to female castrated dogs will augment the maximum tubular secretory capacity but does not influence the creatinine clearance (4). That testosterone propionate is particularly effective as a renotropic agent in rats and dogs which develop compensatory hypertrophy of the remaining kidney after unilateral nephrectomy has been indicated (5). In these animals, the increase in mass of the kidneys was paralleled by an increase in both glomerular filtration rate (inulin clearance) and tubular excretory mass (T_{mp}). The parenteral administration of 25 mgm. of the hormone, daily for 2 weeks, to normal individuals and patients with renal disease failed to increase the renal functions measured.

Despite this last observation, it appeared possible that the administration of testosterone or testosterone propionate might increase the functional capacity of the human kidney if given in amounts comparable (on a weight basis) with those used in animal experiments. A clinical study was undertaken, therefore, to ascertain the effects of large amounts of these steroids on human renal function. The results of that study form the basis of the present report.

METHODS

1. Effective renal flow was ascertained from the plasma clearance (C_{PAH}) of sodium-p-aminohippurate (PAH¹), a compound which has been recommended by Smith and his associates as a substitute for diodrast. During the de-

termination of renal blood flow, the plasma concentration of PAH was maintained at levels of from 1.0 to 3.1 mgm. per cent.

2. The rate of glomerular filtration was measured by the clearance of mannitol (C_M) (7). Plasma levels of mannitol during these observations ranged from 125 to 168 mgm. per cent.

3. The tubular excretory mass was indicated by the clearance of PAH when the plasma concentration of that compound was greater than 66.5 mgm. per cent (T_{mpAH}).

4. The maximal rate of tubular resorption of glucose (T_{mg}) was measured at plasma glucose concentrations above 350 mgm. per cent (8). All determinations were checked both before and immediately after the administration of the hormones.

In a previous communication (9) it was demonstrated that simultaneous measurements of T_{mg} , T_{mpAH} could not be made satisfactorily since the values obtained were effected by the plasma concentration of the test substance. Hence, in the present study, separate periods were used to determine first C_{PAH} , then T_{mpAH} , and finally T_{mg} .

CLINICAL MATERIAL

A total of 9 subjects was studied. Four of these were normal adult males. The first (E. C.) received intramuscularly 90 mgm. of testosterone² in sesame oil daily for 23 days. The second (V. F.) received 100 mgm. per day of testosterone propionate² for a total of 8 days. The third (E. M.) and fourth (D. H.) were injected with 300 mgm. daily of testosterone propionate for 8 and 14 days, respectively.

The fifth subject was a eunuchoid male (M. H.) whose daily excretion of 17-ketosteroids was consistently less than 3 mgm. This subject was included to ascertain whether or not the hypogonadic state affected the renal functions measured. This subject was given 100 mgm. of testosterone propionate per day for 29 days.

The remaining 4 patients investigated each had disorders commonly associated with renal insufficiency. Two had essential hypertension (M. F. P. and J. McG.) and 2 had chronic bilateral pyelonephritis (E. O. and B. H.). The patients with hypertension received daily 100 mgm. of testosterone propionate intramuscularly for 12 and 33 days and those with pyelonephritis each were given 300 mgm. per day for 14 and 16 days.

¹The authors gratefully acknowledge the gift of this material from Sharp & Dohme, Inc., Philadelphia, Pennsylvania.

²The testosterone and testosterone propionate were donated by the Schering Corporation, Bloomfield, New Jersey.

TABLE I

The effects of testosterone and of testosterone propionate on the renal functions of the subjects studied

Subject	Disorder	Period	Daily amount given i.m.	Days	C _M	Tm _{PAH}	Tm _G	C _{PAH}	Blood pressure
			mgm.		ml. per min.	mgm. per min.		ml. per min.	mm. Hg
E. C.	Normal male	Control			114 104	98	232	637 640	120/60
		Testosterone	90	22 23	121 112	89	205	813	120/70 115/70
V. F.	Normal male	Control			119	95	288	711	110/70
		Testosterone propionate	100	9	107	104	310	718	120/70
E. M.	Normal male	Control			165		431	580	90/50
		Testosterone propionate	300	8	143		389	730	100/50
D. H.	Normal male	Control			122		323	650	120/70
		Testosterone propionate	300	14	124		294	740	120/80
M. H.	Eunuchoid male	Control			87 76	66	240	358 375	100/70
		Testosterone propionate	100	14 29	79 86	60 55	190	383	110/60
M. F. P.	Essential hypertension; male	Control			68 77	47	198	265	220/120
		Testosterone propionate	100	12	75	50	217	179	180/110
J. McG.	Essential hypertension; male	Control			64 63	52 46		196 208	200/100
		Testosterone propionate	100	33	62	53		217	180/90
E. O.	Bilateral pyelonephritis; female	Control			30		107	56	170/80
		Testosterone propionate	300	16	31		81	57	160/80
B. H.	Chronic bilateral pyelonephritis; male	Control			52	48	107	212	150/100
		Testosterone propionate	300	14	56	46	107	216	150/100

RESULTS

The essential data are presented in Table I. Before the administration of the steroids, the values obtained for C_M, C_{PAH}, and Tm_G in the normal subjects, the patients with essential hypertension, and those with chronic pyelonephritis agree well with those found by other investigators in similar clinical material (8, 10, 11). However, it is interesting to note that although the eunuchoid male consistently had normal arterial tension and no history of renal disease, the values of C_M, C_{PAH}, and Tm_{PAH} were all abnormally low.

Neither testosterone nor testosterone propionate in the amounts administered apparently had any effect on C_M nor on the Tm_{PAH}. Neither was there obtained any consistent or significant effect of the hormones administered on the C_{PAH} nor the Tm_G.

COMMENT

Maximal renal hypertrophy in the mouse is effected by the administration of 0.1 mgm. per day of testosterone propionate for only 9 days (4). An increase in tubular function in dogs has been observed (4) after the administration of a

single dose of 100 mgm. in 1 case and after 4 daily doses of 100 mgm. each in 2 other animals. On a comparative weight basis, this would indicate about 300 mgm. per day as an effective renotropic dose in man. Hence, had the same relationship existed between administered steroids and renal function in man, the amounts of the compound employed in the present study should have been effective in at least 4 subjects.

Evidently certain steroids do not influence renal functions in general. This has been found true, for example, for alpha estradiol benzoate (12). The administration of this compound appears to be without effect on the clearance of mannitol. It does, however, markedly depress the tubular reabsorption of ascorbic acid. Likewise, in the dog, testosterone propionate apparently does not change the filtration rate but does increase markedly the tubular secretion of diodrast (4). It is possible, therefore, that although the administration of testosterone or testosterone propionate to human beings did not alter the filtration rate nor renal blood flow or TM_{PAH} or TM_G , other renal functions might have been affected. This possibility now is under further investigation.

Whereas the administration of the steroids might not increase renal function in normal subjects above their normal level, the compound might increase depressed function of the diseased kidney. For this reason, patients with impairment of renal function were included, but in these, too, the administered hormones were without measured effect.

CONCLUSION

The administration of testosterone or testosterone propionate in amounts presumed to be adequate for renotropic effects did not alter significantly the rate of glomerular filtration, renal blood flow, the maximum rate of tubular

secretion of p-aminohippurate, or the maximum rate of tubular reabsorption of glucose in 4 normal subjects or in 5 patients with impaired renal function.

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INFLUENZA VIRUS ISOLATIONS AND SEROLOGICAL STUDIES MADE IN BOSTON DURING THE WINTER OF 1943-1944

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Late in March, 1943, a patient was admitted to another hospital in this city and died within a few hours. Autopsy revealed a diffuse hemorrhagic bronchopneumonia and a hemolytic *Staphylococcus aureus* was obtained in pure culture from the pulmonary lesion. A filtered suspension of this lung was used for virus isolation studies in this laboratory. By serial intranasal passage through mice and by intra-allantoic inoculation in chick embryos, it was possible to isolate a virus which was identified as influenza A (1). Two weeks later, a second case was observed which early in its course suggested that of a severe primary atypical pneumonia. The patient died after an illness of 6 days, toward the end of which she exhibited signs and symptoms consistent with an acute myocarditis and the latter was found at autopsy. No significant bacterial pathogen was found in cultures of the lungs of this patient but mouse and chick embryo inoculations of lung suspensions yielded influenza A virus (2).

As far as could be ascertained, influenza-like infections were not particularly prevalent at this time although mild cases may have occurred and escaped recognition. The experience in these cases, however, suggested that influenza A was occurring in sporadic cases. The last known outbreak of influenza A in which a large number of cases were recognized in Boston occurred as part of the more widespread epidemic of this disease in the fall and winter of 1940-41 (3, 4). It was anticipated, however, that another outbreak of influenza might occur during the following season but its nature and extent could not be predicted.

Because of limitations of personnel and facilities and since it was known that extensive studies were being planned by many workers, both in and apart from the armed forces, only limited studies were outlined which would be

more suited to the type of material available in a municipal hospital. One of the chief objectives of the projected study was to determine how readily virus could be isolated and identified by the available methods from typical acute cases of epidemic influenza and from the lungs of fatal cases. Particular interest was also centered about the possible predisposing rôle of influenza virus infections in the severe cases of bacterial pneumonia which occur during and shortly after an epidemic of influenza.

The anticipated epidemic did materialize in this country (5) and in Great Britain (6) and possibly in many other countries. Most of the cases in Boston occurred during the first 2 weeks of December 1943. The studies made during the height of this epidemic and for several weeks after it had subsided yielded results that are of some interest and they are reported in this paper.

PATIENTS, MATERIALS, AND METHODS

Patients. The 4 epidemic cases chosen for isolation of virus from throat washings were admitted to the Boston City Hospital between December 3 and 14. They were considered clinically to be characteristic cases and were selected because they were still acutely ill, with a fever of 102° F. or higher, and had had symptoms for 2 days or less at the time when the throat washings were obtained. Influenza virus was also isolated from the lung in a fifth case in which death occurred on December 13, 3 hours after admission to the Evans Memorial Hospital.¹

Among the cases which are being classified as post-epidemic, the first to be studied from which a virus was isolated, was admitted to the Boston City Hospital on January 31, 1944, and died a few hours later of a diffuse bronchopneumonia. Viruses were also isolated from 6 other clinical cases and in 1 other fatal case, all admitted between February 8 and March 12.

Cases chosen for serological studies included: (1) The clinical cases from which virus isolations were accomplished. (2) Similar patients who were admitted during the height of the epidemic and who were ill more than

¹ Materials obtained through the kindness of Drs. Chester S. Keefer and John J. Curry.

3 days or were already afebrile at the time when they were first seen, so that virus isolations were not attempted. (3) Patients with severe bacterial pneumonia occurring during the height of the epidemic and in the following few weeks. The majority of them were on the medical wards in the middle of January; most of them had extensive pulmonary lesions and many responded poorly to chemotherapy. Each of them gave a history of clinical influenza sometime after the middle of November. (4) A group of patients who were on the wards at the same time for various conditions other than acute respiratory infections but who had had symptoms of an influenza-like infection after the middle of November. (5) Cases similar to the latter whose earlier symptoms were interpreted as those of common cold and not influenza. Most of them had had predominantly coryza without fever or constitutional symptoms. (6) Similar patients and some hospital staff members who had no symptoms either of a common cold or of influenza after October 1943. A few cases of pneumonia are included in the last 2 groups, but very few cases of severe pneumonia were encountered at this time in which there was no history of influenza during the epidemic.

Virus isolations. In the early acute cases, the patients were asked to gargle thoroughly 3 times with 30 ml. of sterile broth containing 10 per cent horse serum. The washings were cultured and were then divided into 2 parts, 1 of which was passed through a Berkefeld "V" or a mandrel filter. These were used for inoculations immediately or were stored in a carbon-dioxide freezing box for later inoculations.

White mice, in groups of 4 to 6, were each inoculated intranasally under light ether anesthesia and passages of 20 per cent pooled lung suspensions were done at 3- to 5-day intervals. Only the shorter intervals were used in the latter part of the study. After pulmonary lesions began to appear, or if the mice died with extensive and characteristic lesions, subsequent passages were made with 1:10 to 1:1000 dilutions of the lung suspensions, depending on the extent of the lesions or the time of death. The presence of the virus was recognized by the characteristic lesions. The virus was then identified by neutralization tests in mice with immune rabbit or ferret serums² prepared against the PR8 (7) strain of influenza A and the Lee (8) strain of influenza B. In some instances, this was corroborated by demonstrating resistance to infection with lethal doses of these standard strains in mice which had previously survived sublethal infections with the patient's virus. Further identification of some of the mouse passage viruses was done by inoculating sterile filtered suspensions into chick embryos, as detailed below, and identifying the virus harvested from the allantoic fluid by agglutination of hen's cells and by the specific inhibition of this agglutination with immune rabbit sera (9).

Direct isolations were also made in chick embryos by adapting the methods of Hirst (10 to 12). The unfiltered or filtered washings, or both were inoculated in 0.1 ml. amounts into the chorioallantoic sac of 11- to 12-day-old

chick embryos (3 to 6 eggs each) and the allantoic fluid was harvested after 48 hours additional incubation at 37° C. The presence of influenza virus was indicated by the agglutination of the red cells of the embryo which were permitted to escape from the ruptured vessels before the harvesting. When there was little or no agglutination visible, the fluid was allowed to stand in an ice bath for 1 to 2 hours in order to adsorb the virus on to the red cells. The supernatant fluid after centrifugation was then removed. A small amount of 10 per cent horse serum broth or of the supernate was added to the sedimented red cells from which the virus was then eluted by incubation for 2½ hours at 37° C. with frequent shaking. The cells were then removed by centrifugation and the supernatant serum broth or allantoic fluid containing the virus was used for further passages. When there was a large amount of virus as indicated by strong and immediate agglutination of the red cells as the allantoic fluid was removed, suitable dilutions of the virus up to 1:100,000 were used for later passages. When the allantoic fluid was contaminated as evidenced by the dark discoloration or hemolysis of the red cells, the adsorption and elution were followed by filtration before further passages were made. The quantity of virus contained in the allantoic fluid was titrated by agglutination of serial dilutions with hen's cells and it was identified by inhibition of this agglutination with type-specific A and B immune sera (13).

Serological tests. These consisted of complement fixation (14) and agglutination-inhibition (9, 13) tests. The antigens used consisted of influenza A (PR8) and B (Lee) as well as the patients' homologous viruses each contained in allantoic fluid of chick-embryo passages. Serial samples of acute and convalescent sera were obtained from the patients in Groups 1 and 2 (see above) and individual or multiple samples of serum were obtained from the remaining patients. The titers are expressed as the reciprocal of the greatest initial dilution of serum giving 25 to 50 per cent hemolysis in the complement fixation tests and almost complete inhibition (0 to 1+ agglutination) in the Hirst tests. The complement fixation tests were carried out in the laboratories of the Department of Bacteriology and Immunology of the Harvard Medical School. We are indebted to Dr. John F. Enders for advice and for the use of his facilities and to Mrs. Jeannette Levens and Miss Elizabeth G. Mills for technical assistance.

ISOLATION OF VIRUSES FROM EPIDEMIC CASES

Attempts were made to isolate and identify a virus from materials obtained during the height of the epidemic in 5 cases, including 1 fatal case. Throat washings were obtained during the first or second day of the disease in the living cases and a suspension of lung was obtained at autopsy from the fatal case and used for intranasal inoculations of white mice and for intra-allantoic inoculation in chick embryos. Each of the

² Serums obtained through the kindness of Dr. Frank L. Horsfall, Jr.

patients who furnished the throat washings had a fever of 102° F. or higher at the time and the illness in each case was of more than average severity. In Case 1 and Case 3, the influenza was apparently uncomplicated except for transient acute sinusitis in the former. In both of these cases, the fever subsided and the patient was definitely improved about 24 hours after the material was obtained. The other 3 patients had, or later developed complicating pulmonary infections. A resumé of these 3 cases follows.

Case 2. A 15-year-old colored boy was admitted to the Boston City Hospital in a comatose condition on the morning of December 4. From his mother it was learned that he had been well until the morning of December 2 when he complained of a "head cold" and stayed home from school. He apparently had had severe headache and chilly sensations but no coryza. During that day and the next, he felt increasing weakness and prostration and asked not to be awakened early the following day. On the morning of December 4, however, his mother found him comatose in bed, breathing rapidly, with bloody frothy material oozing from his mouth and nose and she sent him into the hospital.

The latter findings were still present when the patient reached the ward. His temperature then was 104° F., pulse 136, and respiration 60 and shallow. There was slight dullness bilaterally and showers of medium and coarse moist râles were heard throughout both lungs. The rest of the physical examination was essentially negative. The leukocyte count was 4650 with 65 per cent polymorphonuclear neutrophils, 33 per cent lymphocytes, and 1 per cent each of monocytes and basophiles. X-ray of the chest showed both lung fields diffusely infiltrated with small fluffy areas of density.

Much of the material from the nose and throat was removed by suction and the patient was given oxygen therapy soon after entry. He was then given full doses of sulfadiazine starting with 5 grams of its sodium salt, intravenously. This dose was repeated in 12 hours, after which oral dosage of 1 gram every 4 hours was continued for another week. Improvement began within a few hours, and the patient was afebrile and conscious the following day. He remained apathetic for another 2 or 3 days, after which his recovery was steady and complete. The physical signs in the lungs cleared rapidly but there was still some residual infiltration in the bases of the lungs visible by x-ray on December 11.

The nasopharyngeal washings which were collected shortly after entry and contained some of the bloody and frothy exudate were used for virus studies. Culture of this material showed pneumococcus type 18 in moderate numbers.

Case 4. A 24-year-old nurse began to have chilly sensations, malaise, headache, and a general aching feeling on the morning of December 13. At the time of admission to the ward on December 14, her temperature was 102° F.,

pulse 96, and respirations 24, and she felt markedly prostrated. Except for a slightly injected throat, examination was essentially negative. Culture of the throat washings obtained for virus study at this time yielded a scant growth of *Staphylococcus aureus*. The white blood count was 6700 with 84 per cent polymorphonuclear neutrophils.

After a few hours, the patient's fever subsided and she felt somewhat improved. During the next day, however, the temperature rose steadily to 103.8° and the pulse to 140 and the patient again became markedly prostrated. A severe cough with presternal and left lower chest pain began at this time and increased gradually in severity. Sputum was scant at first and became increasingly purulent and copious, with occasional admixtures of blood. Signs of diffuse bronchitis and of bronchopneumonia involving chiefly the left lower lobe became evident and the latter was confirmed by x-ray.

Chemotherapy with full oral doses of sulfapyrazine was started at the time of the recurrence of the fever and was continued until January 5. The patient began to improve after 2 days of this therapy although low grade fever, sputum, and signs of a small amount of fluid in the left pleura and of consolidation and possibly abscess formation of the left lower lobe persisted for 2 weeks. After that time, the patient remained somewhat weak for several days, but cough and pain subsided and the lungs cleared both to physical and x-ray examinations. Many specimens of sputum each yielded a copious growth of *Staphylococcus aureus* in almost pure culture, but cultures of the blood all failed to show any growth.³

Case A-1. An 18-year-old female office worker was admitted to the Evans Memorial on December 13. Her illness began 2 days previously with the complaint of sore throat and slight dysphagia. On the following day, she had anorexia, nausea, and vomiting and she became drowsy and prostrated. Early on the morning of admission, she developed extremely severe pain across the lower chest, a productive cough, and rapidly increasing delirium. In this state, she was sent to the hospital.

On arrival, her temperature was 106° F., pulse 175, respirations 53, and blood pressure 60/40. She appeared critically ill, toxic, markedly cyanotic, irrational, irresponsible, and incontinent of urine and feces. She was dehydrated and had thick ropy inspissated mucopurulent material at the base of her tongue. There was a deep red diffuse injection of the pharynx. In the lungs, there were signs of patchy consolidation of the right lower lobe and this was confirmed by x-ray which also showed some fine mottled infiltration of the middle of the left lung field. The white blood count was 1350 with 24 per cent polymorphonuclear neutrophils, 72 per cent lymphocytes, and 4 per cent monocytes. Sputum and throat cultures showed beta hemolytic streptococcus and *Staphylococcus aureus* and the latter was also obtained from the blood culture.

The patient failed to respond to treatment with oxygen and parenteral injections of sulfadiazine, glucose solutions, and various stimulants. Consolidation of the lungs spread

³ A chart showing details of the clinical course and therapy in this case is shown elsewhere (15).

TABLE I

Isolations of viruses from cases of clinical influenza during and after the epidemic period

Case no.	Date of onset	Virus isolation		Unfiltered		Filtered		Type
		Date	Source	Mouse	Egg	Mouse	Egg	
Epidemic strains								
1	Dec. 2	Dec. 3	Throat	+	-	+	+	A
2	Dec. 2	Dec. 4	Throat	+	+	+	+	A
3	Dec. 5	Dec. 7	Throat	-	+	+	+	A
4	Dec. 13	Dec. 14	Throat	+	+	-	-	?A
A-1	Dec. 11	Dec. 13	Lung	-	-	0	+	?A
Post-epidemic strains								
66	Feb. 2	Feb. 8	Throat	+	-	-	+	?A
67	Feb. 7	Feb. 11	Throat	+	+	-	+	?A
68	Feb. 26	Feb. 29	Throat	+	+	-	+	?A
69	Mar. 5	Mar. 12	Throat	+	+	-	+	?A
70	Mar. 6	Mar. 8	Throat	+	0	-	0	?A
71	Mar. 11	Mar. 12	Throat	+	+	-	+	?A
A-2	Jan. 23	Feb. 1	Lung	-	-	+	+	A
A-3	Mar. 4	Mar. 11	Lung	-	-	+	+	A

+ = Virus isolated; 0 = Failure; - = Not attempted.

rapidly and the patient soon developed muscular twitches and mild convulsions. She died about 3 hours after admission. Autopsy showed diffuse acute hemorrhagic bronchopneumonia. Hemolytic streptococcus and *Staphylococcus aureus* were obtained from all parts of the lung, but not from the cardiac blood. Some of this lung was used for the virus studies.

The results of the virus isolation studies in these 5 cases are summarized in the upper portion of Table I and the bacteriological findings are summarized in Table II. Viruses were obtained by direct inoculation of unfiltered throat washings into mice in Cases 1, 2, and 4, and into chick

embryos in Cases 2 and 4. In the latter, the allantoic fluids harvested from the first eggs were filtered and further passages were carried out with the filtrates. Mouse-lung suspensions were also filtered before further passage when there was bacterial contamination, but this was no more frequent in mice originally receiving unfiltered lung than in mice which received filtered washings.

Similar mouse and egg inoculations were done with filtered throat washings in Cases 1 and 3 and with the filtrate of the lung suspension of Case A-1. Viruses were obtained in each of these attempts with one exception, namely the mouse inoculations of the lung filtrate of Case A-1.

The presence of virus was noted in the allantoic fluid following the primary inoculation in every instance. This was evidenced by the presence of various degrees of agglutination of the chick cells which were allowed to mix with the allantoic fluid by rupturing the blood vessels before the fluid was harvested. In most instances, the virus contained in this allantoic fluid was concentrated by adsorption on to these cells and eluted into a smaller volume of the supernate, or of serum broth, for subsequent passages. This procedure was sometimes followed also in later egg passages. Whenever there was moderate or marked agglutination, however, the eluent was diluted for subsequent passages.

In mice, characteristic lesions were noted in the lungs in the second or third mouse passage

TABLE II
Predominant organisms in cases of influenza

Epidemic influenza			Post-epidemic cases		
Case	Throat	Sputum	Case	Throat	Sputum
1	Str. α; S. au.		66	Str. α	
2	Pn. 18	Pn. 18	67		Pn. 13; Str. α
3	Str. α		68		Pn. 28; Str. α
4	S. au.	S. au.	69	Str. α	Pn. 9
5	Str. β	Str. β	70	Str. β	
7	Str. β; S. au.		71	Str. β	
8	Pn. 7	Pn. 7	72		Str. α
9		Pn. 8, Pn. 20	74		Pn. 9
13	Str. α		A2		Str. α*
A1	S. au.; Str. β	Str. β; S. au.*	A3	Pn. 1	Pn. 1*

Str. = Streptococcus; S. au. = Staphylococcus aureus; Pn. = Pneumococcus.

* Same organisms obtained from lungs at autopsy. Blood cultures were sterile in each instance except in Case A1, S. au. was cultured from the blood before death and in Case A3 Pn. 1 was obtained from the blood during life and from cardiac blood taken at autopsy.

in each instance and deaths began to occur in 3 to 5 days in the mice of the third to the fifth passage. The results of both the mouse and egg inoculations were similar with the filtered and with the unfiltered washings. In the mice inoculated with the filtered suspension of lung from Case A-1, lesions were not obtained after 6 passages and further attempts were not made.

The viruses obtained in Cases 1, 2, and 3 from both mice and chick embryos were readily indentified as strains of influenza A similar to PR8. Neutralization tests in mice were carried out with antisera prepared in ferrets and rabbits against the PR8 strain of influenza A and the Lee strain of influenza B. The influenza A serum afforded complete protection against 100 to 1000 lethal doses of these strains while control mice receiving the virus alone and those receiving the anti-B serum together with the same amounts of virus all died with typical lesions. The agglutination of hen's cells by the strains obtained in the allantoic fluid in these cases was inhibited by influenza A antiserum in high titer and only in low titers by influenza B antisera. Rabbit sera were used for the latter titrations.

The egg-passage strains in Cases 4 and A-1 gave only slightly higher titers of agglutination inhibition with anti-A serum than with anti-B serum, and thus differed from the previous strains. The results of neutralization tests in mice with the strains from these 2 cases, however, gave results similar to those obtained in the first 3 cases. Mice were protected from fatal infection with these strains by anti-PR8 but not by anti-B serum. Further studies of these and other strains are now being carried out in collaboration with Dr. John F. Enders and Miss Elizabeth G. Mills.

SEROLOGICAL STUDIES IN CASES OF EPIDEMIC INFLUENZA

Blood for serological studies was obtained at the time of the throat washings and at intervals thereafter in Cases 1 to 4. Serial samples of serum were also collected from 7 other patients, numbers 7 to 13, who were admitted to the Boston City Hospital with characteristic symptoms of influenza which began between December 1 and 23. They had all been acutely ill and febrile within 1 or 2 days of the time the first

blood was obtained, but the onset of influenza had occurred up to 15 days previously. Cases 5 and 6 are from other hospitals and represent patients who had physical and x-ray signs of atypical pneumonia which followed symptoms of influenza. The latter began on November 14 in Case 5 and on November 24 in Case 6. Except as already mentioned, there were no complications in any of these cases other than the finding of a few scattered crepitant râles which were heard in every case.

The results of the serological tests with the PR8 strain of influenza A in the 13 cases and with the patients' homologous strains in Cases 1 to 4 are given in Table III. In 11 of the patients the initial serum was obtained 6 days or less after the onset of the influenza. The titers of complement fixing antibodies against PR8 in these initial sera were less than 4 in 4 cases, 8 in 5 cases, and 16 in 12 cases. The first serum in Case 5 was obtained 10 days after the onset and had a titer of 128 and the first serum in Case 13, obtained on the fifteenth day had a titer of 512.

Subsequent sera all showed a significant rise in titer. In Case 12, there was a 4-fold rise between the fifth and eleventh days and a similar rise from the initial high titer occurred between the fifteenth and the eighteenth day in Case 13. In 3 other cases, including Case 5, there was an 8-fold rise in titer and the remaining cases all showed rises which were considerably greater.

The maximum complement fixation titer demonstrated in Case 12 was 32. This was in a serum obtained on the eleventh day which was the last day of observation. In Case 6, the maximum titer was 64 at the time of the last observation made on the thirteenth day. In the remaining cases, the maximum titers ranged from 128 to 2048 and titers of 512 or higher were obtained in 6 cases. Of interest is the demonstration of 4- to 8-fold rises in titer after the end of the third week in Cases 3, 5, and 7. While no special effort was made to determine how long the elevated titers persisted, it is worth noting that titers of 64 to 512 were still present in sera obtained 10 to 12 weeks after the onset of influenza in several cases.

The titers of the agglutination-inhibition tests with PR8 roughly paralleled those of the comple-

TABLE III

Results of serological tests in cases of clinical influenza observed during the course of the epidemic

Case no.	Date of onset	Date of serum	Days after onset	Titer vs. PR8		Titer of A.I. vs. own strain
				C.F.	A.I.	
1	Dec. 2	Dec. 3	1	16	16	8
		Dec. 7	5	32	8	8
		Dec. 24	22	128	256	64
		Jan. 31	60	64	128	128
		Feb. 28	88	64	64	256
2	Dec. 2	Dec. 5	3	<4	4	<4
		Dec. 13	11	256	256	512
3	Dec. 5	Dec. 7	2	<4	4	4
		Dec. 31	26	128	256	128
		Jan. 17	43	2048	512	512
		Feb. 28	85	512	256	256
4	Dec. 13	Dec. 14	1	<4	8	<4
		Dec. 20	7	64	256	16
		Dec. 28	15	1024	512	
		Jan. 17	35	1024	512	256
		Feb. 28	77	512	256	128
5	Nov. 14	Nov. 24	10	128	256	
		Nov. 27	13	128		
		Dec. 2	18	256		
		Dec. 7	23	256		
		Dec. 10	26	1024	512	
		Dec. 20	37	512	256	
6	Nov. 24	Nov. 30	6	8	8	
		Dec. 7	13	64	64	
7	Dec. 1	Dec. 7	6	8	8	
		Dec. 12	11	32	32	
		Dec. 28	27	64	128	
		Jan. 17	47	128	128	
		Feb. 21	82	256	128	
8	Dec. 5	Dec. 6	1	8	8	
		Dec. 13	8	128	32	
		Dec. 23	18	128	32	
		Feb. 1	58	64	128	
		Mar. 1	87	64	128	
9	Dec. 6	Dec. 9	3	8		
		Jan. 4	28	512	256	
10	Dec. 12	Dec. 14	2	<4	4	
		Dec. 23	11	512	256	
		Feb. 21	71	256	32	
11	Dec. 15	Dec. 16	1	16	16	
		Dec. 30	15	128	256	
		Jan. 17	33	256	128	
		Feb. 22	69	256	128	
12	Dec. 17	Dec. 22	5	8	4	
		Dec. 28	11	32	64	
13	Dec. 23	Jan. 7	15	512	512	
		Jan. 10	18	2048	512	
		Jan. 21	29	512	512	

C.F. = Complement fixation; A.I. = Inhibition of red cell agglutination (Hirst). Dates: Nov. and Dec. 1943; Jan. and Feb. 1944. The patients' own virus strains were isolated on the day the first serum was obtained.

ment fixation tests. The titers of the individual sera were either the same or showed a 2-fold difference with the latter test usually giving the higher titers. Only occasional sera showed a 4-fold difference. In Cases 1 to 4, the Hirst inhibition titers with the homologous strains showed similar marked rises. There were minor differences, however, in the results of tests with the 2 viruses in individual serum samples.

SEROLOGICAL STUDIES IN OTHER GROUPS OF CASES

In relation to the influenza epidemic of 1940-41, Pearson *et al.* (3) obtained sera from patients with bacterial pneumonia, admitted to the Boston City Hospital during and after the height of the epidemic. Serological evidence was obtained from these cases which indicated that a large number of them had had contact with influenza A virus and suggested a possible relation between the 2 diseases. During the height of the present epidemic and for a few weeks thereafter, it was noted that bacterial pneumonias were occurring with more than the expected frequency and the cases appeared to be unusually severe. Similar increases in the incidence of pneumonia and of its severity, as indicated by a higher mortality, were also noted elsewhere (5). It was, therefore, of some interest to study the serum of patients admitted to the hospital for pneumonia after this epidemic in order to obtain evidence of the rôle being played in these cases by the influenza A virus which was obviously the predominant agent during the epidemic.

Almost all of the patients with severe pneumonia observed during the last half of January gave a history of an influenza-like infection during the epidemic. Sera were, therefore, also obtained from another group of patients who did not have pneumonia but gave a history of symptoms of influenza during the epidemic. The results in the latter cases will be presented first.

ANTIBODIES FOR INFLUENZA A OBTAINED AFTER THE EPIDEMIC IN CASES WITHOUT PNEUMONIA BUT WITH A HISTORY OF INFLUENZA DURING THE EPIDEMIC

Sera were obtained between January 17 and February 16 from 13 patients who were admitted

for a variety of conditions other than pneumonia, but had a history of influenza-like symptoms between December 1 and January 10. Single samples were obtained in 10 of the cases and 2 or 3 samples in each of the other 3 cases. The interval between the onset of the influenza and the time the first or only serum was obtained was less than 3 weeks in 3 cases, 26 to 37 days in 5 cases, and 44 to 56 days in the remaining 5 cases. The results are shown in Table IV.

TABLE IV

Antibodies for influenza A (PR8) in serums obtained after January 15, 1944 from persons giving a characteristic history of clinical influenza during the epidemic (Dec. 1943 and early in Jan. 1944)

Case no.	Date of onset	Date of serum	Days after onset	Titer vs. PR8	
				C.F.	A.I.
14	Dec. 1	Jan. 22	52	16	8
15	Dec. 5	Jan. 22	48	128	64
16	Dec. 5	Jan. 24	50	128	64
17	Dec. 11	Jan. 24	44	<16	<4
18	Dec. 15	Feb. 9*	56	16	<4
		Feb. 16	63	64	64
19	Dec. 18	Jan. 24	37	128	64
20	Dec. 24	Jan. 22	29	64	32
21	Dec. 26	Jan. 21	26	128	64
22	Dec. 27	Jan. 22	26	64	32
23	Dec. 28	Jan. 28	31	512	128
		Jan. 31	34	128	
		Feb. 10	44	128	256
24	Jan. 4	Jan. 24	20	64	16
25	Jan. 4	Jan. 17	13	128	64
26	Jan. 10	Jan. 21	11	128	64
		Jan. 29	19	64	64
		Feb. 1	22	128	64

* The patient was admitted on this date for a recurrence of symptoms similar to those of her earlier attack of influenza in December.

In 3 cases, the titer of complement fixation with PR8 was 16 or less. In each of these cases, the serum was obtained more than 6 weeks following the onset of influenza. In 1 of them, Case 18, the patient was admitted on February 9 for a recurrence of symptoms similar to those of her original attack of influenza on December

15. A second serum obtained 1 week later showed a titer of 64, suggesting either a response to the second infection or an anamnestic reaction. In 9 of the other 10 cases, the titers were 64 or 128.

In Case 23, the titer of the first serum obtained 1 month after the influenzal infection was 512 and 2 subsequent samples each had a titer of 128. It is of interest that this patient entered the hospital for a *Staphylococcus aureus* septicemia and probably had endocarditis but the original focus could not be determined. The portal of entry may well have been the respiratory tract and the original staphylococcal infection, though symptomless, may have been related to the earlier influenzal infection.

The results of the agglutination-inhibition tests with PR8 roughly paralleled those of the complement fixation tests as in the previous cases. Most of the titers, however, showed a 2-fold difference in favor of the latter test.

These findings suggest that, following a widespread epidemic of influenza A, significant titers of antibodies against the epidemic type can be demonstrated in most persons who give a history of having had symptoms of influenza during the epidemic. The antibodies in the present cases were demonstrable for as long as 6 to 8 weeks after the onset of symptoms in many instances.

ANTIBODIES FOR INFLUENZA A IN CASES OF BACTERIAL PNEUMONIA HAVING A HISTORY OF CLINICAL INFLUENZA DURING THE EPIDEMIC

Sera were obtained from 24 patients who were in the hospital for severe pneumonia during and after the epidemic of influenza. These patients all gave a history of having symptoms of influenza during the epidemic. The results of the serological tests and some of the relevant data in these cases are summarized in Table V.

The onset of the symptoms of influenza in these cases occurred between November 24 and January 7 and the pneumonia began between December 4 and March 1. The interval between the onset of the 2 diseases varied. In 11 cases, the pneumonia began at about the same time or within 5 days after the influenza; in 7 others, the interval was 7 to 16 days; while in the remaining 6 cases, the onset of the pneumonia

TABLE V

Antibodies for influenza A (PR8) in cases of severe bacterial pneumonia occurring during and after the epidemic in patients with characteristic history of clinical influenza during the epidemic

Case no.	Date of onset of influenza	Date of serum	Days after onset of influenza	Titer vs. PR8		Relevant facts concerning the pneumonia			
				C.F.	A.I.	Onset	Days after influenza	Organism	W.B.C.
27	Nov. 24	Jan. 18	55	512	512	Dec. 31	37	S. au.	6.5-10.0
28	Nov. 30	Dec. 11	11	128	64	Dec. 4†	4	Pn. 1(+)	4.5-20.6
29	Dec. 1	Jan. 18	49	256	128	Jan. 1	31	Str. α	5.2-5.4
30	Dec. 11	Jan. 17	37	512	512	Dec. 11	0	Pn. 23*	10.0-11.4
31	Dec. 12	Jan. 18	37	256	512	Jan. 12	31	Str. α	9.3-10.3
32	Dec. 12	Dec. 22	10	256	256	Dec. 15	3	Pn. 17	2.7-5.4
33	Dec. 15	Feb. 9	56	128	128	Jan. 25	41	Pn. 8	15 -22
34	Dec. 18	Jan. 17	31	256	64	Dec. 25	7	Neg.	22
35	Dec. 18	Jan. 18	31	256	64	Jan. 1	13	Str. α	6.0-13.2
36	Dec. 19	Jan. 11 Jan. 17	23 29	128 128	128 64	Dec. 19	0	Pn. 25(+)	4.5-15.0
37	Dec. 20	Dec. 29 Jan. 11 Jan. 17 Feb. 9	9 22 28 51	64 128 128 64	64 128 128 64	Dec. 25	5	S. au. (+)	5.6-14.9
38	Dec. 20	Jan. 18	29	512	1024	Dec. 28	8	S. au.*	12 -14
39	Dec. 20	Jan. 17	28	128	128	Jan. 13	24	Pn. 23	16
40	Dec. 21	Jan. 11 Jan. 18	21 28	1024 1024	1024 1024	Dec. 22	1	S. au. Pn. 3	8.2-25
41	Dec. 23	Jan. 17	25	512	256	Dec. 30	7	S. au.	4.1-7.2
42	Dec. 23	Jan. 17	25	32	32	Dec. 26	3	Pn. 7(+)	6.4-15.4
43	Dec. 25	Jan. 17	23	64	64	Jan. 10	16	Pn. 2(+)	4.0-7.5
44	Dec. 25	Jan. 18	24	128	64	Dec. 28	3	Pn. 1	9.2-28
45	Dec. 27	Jan. 17	21	64	64	Jan. 10	14	Pn. 25	19 -24
46	Dec. 27	Jan. 18	22	128	64	Dec. 28	1	Pn. 7(+)	5.6-11.5
47	Jan. 2	Jan. 18	16	128	64	Jan. 4	2	Pn. 3(+)	5.6-14
48	Jan. 2	Jan. 15 Jan. 18	13 16	64 64	64 32	Jan. 10	8	S. au.	15
49	Jan. 3	Mar. 7	64	32	32	Mar. 1	58	Str. α	8.0-10
50	Jan. 7	Jan. 18	11	64	64	Jan. 8	1	Str. α	6.4-12.6

† Died Dec. 11. Virus isolation from the lung attempted but failed.

(+) Same organism obtained from blood culture.

* Sputum positive for tubercle bacilli in convalescence.

The months Nov. and Dec. are in 1943, Jan. to Mar. are 1944.

C.F. = complement fixation; A.I. = inhibition of hen's red cell agglutination (Hirst).

S. au. = *Staphylococcus aureus* (hemolytic and coagulase positive).

Str. α = *Alpha hemolytic streptococcus*; Pn. = *pneumococcus* (Number represents the type).

W.B.C. = Range of total white blood count during the febrile period (Thousands).

occurred from 24 to 58 days after the first symptom of influenza.

The predominant organism obtained during the pneumonia was a pneumococcus in 12 of the cases, *Staphylococcus aureus* in 5 cases, and both a pneumococcus and staphylococcus in 1 case. Positive blood cultures were obtained in 6 of the pneumococcal pneumonias and in 1 of the staphylococcal pneumonias. In 5 cases, the predominant organism was an alpha hemolytic streptococcus and no significant pathogen was recovered from 1 case. In these last 6 cases, the signs and symptoms did not differ materially from those of the other cases. All of them, however, had received sulfonamide therapy before the materials were obtained for culture and this may have accounted for the failure to obtain more significant pathogens which may have been present earlier and accounted for the pulmonary lesions.

Of interest was the finding of leukopenia or of low normal leukocyte counts in most of these cases during the height of the febrile stage of the pneumonia. Leukocyte counts below 5000 were obtained in 5 cases, between 5000 and 6500 in 8, and between 7800 and 10,000 in 4 cases. Polynuclear neutrophils with many immature forms predominated in all these cases. Higher counts were noted later in the course of the acute illness in some of the cases.

Single specimens of serum were obtained in most of these cases during the third week in January while a few were obtained at other times. In 4 cases, additional sera were obtained after a few days. The interval between the onset of the influenza and the time the serum was obtained varied from 9 to 64 days and was more than 31 days in 6 cases.

The titers of influenza A antibodies in these cases were comparable to those obtained during convalescence in the influenza cases studied during the epidemic. They were generally higher than those obtained in the cases with a history of influenza but without pneumonia. The intervals between the influenza and the serum collections were somewhat longer in the latter group of cases, but this probably did not account for the difference, since some of the later sera in the pneumonia cases showed somewhat higher titers.

A titer of 32 was obtained by the complement fixation and agglutination-inhibition tests in 2 cases. In one of them, the serum was obtained on the 64th day after the influenza, and in the other, it was obtained on the twenty-fifth day after the influenza and early in the course of a severe bacteremic pneumonia. Of the remaining cases, the sera of 12 had titers of 64 or 128 by the complement fixation test and 10 had titers of 256 or higher. The agglutination-inhibition titers were very similar to those obtained by the complement fixation tests. The differences in titers of the same sera by the 2 tests were similar to those obtained in the 2 previous groups of cases. Most of the titers were the same or showed a 2-fold difference and only an occasional serum showed a 4-fold difference in titer with the 2 tests.

The serological findings in these cases thus corroborated the clinical histories. They indicate that these patients with severe pneumonia, many of whom responded less favorably than usual to intensive sulfonamide therapy, had had recent infections with influenza A. They suggest further that the influenzal infection may have been a factor not only in the pathogenesis of these pneumonias but also in their severity.

INFLUENZAL ANTIBODIES IN PERSONS WITHOUT A HISTORY OF CLINICAL INFLUENZA

In the latter half of January when most of the sera from the latter group of pneumonia patients were obtained, a search was also made for other patients with severe pneumonia who denied having had, during the epidemic, symptoms which could be interpreted as those of influenza. Very few such cases were found on the wards at the time but some were encountered subsequently. Sera were obtained from a few such cases and from a number of others without pneumonia. These served, in a way, as controls for the previous 2 groups of cases and supplied further data concerning the significance of the antibody titers in relation to the history of influenza which was obtained. The hospital patients and personnel from whom the sera were obtained fell into 2 groups; one included those from whom a history was elicited of a recent simple upper respiratory tract infection consisting mostly of coryza and cough without

constitutional symptoms, or of acute follicular tonsillitis; the other group consisted of persons who denied any symptoms of respiratory tract infections since the outbreak of the epidemic.

INFLUENZAL ANTIBODIES AFTER SIMPLE UPPER RESPIRATORY TRACT INFECTIONS

Sera were obtained on one or more occasions from 15 persons who gave a history of having had, between November 15 and February 12, symptoms of upper respiratory tract infection without the constitutional symptoms of influenza. Included were 5 patients with pneumococcal pneumonia of whom 3 had positive blood cultures and 1 died. In 1 additional case, alpha hemolytic streptococcus was obtained in cultures of the sputum during a typical attack of lobar pneumonia although its etiological rôle is doubtful. Among the others were 3 hospital staff members, 1 of whom had had follicular tonsillitis, and 6 patients who were in the hospital for a variety of conditions, including another case of acute hemolytic streptococcal tonsillitis. Virus isolation was attempted in the latter case and was unsuccessful.

No significant titers or rises in titer of antibodies for influenza A were found in any of these sera. A complement fixation titer of 32 was obtained in 6 cases including 2 in which a previous serum had a titer of 16. The titers in the rest of the cases were 16 or less. Comparable results were obtained with the Hirst inhibition test. As in the previous cases, the titers by the 2 tests in the individual sera were in close agreement.

RESULTS IN PERSONS WITHOUT PREVIOUS UPPER RESPIRATORY TRACT INFECTION

Sera of 26 persons who denied having any symptoms of acute upper respiratory tract infections during the previous 3 months were also tested. Most of these sera were obtained between January 6 and 24. Included were sera from 4 patients with severe pneumonia and from several members of the hospital and laboratory staff. The maximum complement fixation titer was 128 in 2 instances and 64 in 2 others, and, in each of these 4 cases, the agglutination-inhibition titer was 64. The titers in all the remaining cases were 32 or less by either test.

Here again, the titers in the individual sera obtained by the complement fixation and agglutination-inhibition tests were in essential agreement. In Case 80, an 8-fold rise in titer to 64 was noted between December 6 and January 17 although no symptoms of infection had occurred in the intervening period. This person handled all of the virus-infected materials that were brought in from the wards for this present study and carried out all of the influenza virus isolations. None of those who studied the patients on the wards and obtained the materials for the isolations had any significant titers or rises in titer. Three of the cases of influenza, however, were in hospital interns.

INFLUENZAL ANTIBODIES IN POST-EPIDEMIC CASES OF INFLUENZA

A few of the sera in the last 2 groups were obtained in February and early in March. At first, it was felt that the epidemic had subsided early in January, since typical cases of influenza were not being encountered after that time. On February 1, however, evidence was obtained that influenza might still be occurring sporadically. On that day, a patient (Case A-2) died after only a few hours in the hospital and, at autopsy, showed an acute hemorrhagic bronchopneumonia from which no significant pathogen could be obtained. Influenza virus, however, was readily isolated from a filtrate of this lung. Subsequently, several other cases of clinical influenza were admitted to the hospital and strains of influenza virus were isolated from 6 clinical cases, and from the lung of another fatal case, in which the symptoms of influenza began between January 23 and March 11.

The symptoms in most of these cases were quite characteristic of influenza but the subsequent course in 3 of them was atypical. The histories of these 3 cases will, therefore, be presented briefly.

Case 66. The patient was a 45-year-old cook who was admitted to the hospital on February 7. He had been in very good health until 1 week previously when he had slight coryza without constitutional symptoms. On February 6, he suddenly developed severe headache, generalized aches and pains in his bones and muscles, and frequent chills followed by fever. There was slight cough and yellowish mucoid sputum. The admission temperature was 102° F., pulse 70, and respiration 25. Except

for a few râles in the left lower chest, physical examination was essentially negative. The leukocyte count was 4700 on that day and 7800 the next day and there was a predominance of mature polymorphonuclear neutrophils. On symptomatic therapy the patient's temperature dropped gradually to normal in about 36 hours, but 24 hours later, the temperature again rose and thereafter assumed a swinging character, ranging from 99 to 101 or 102° daily, with pulse remaining below 80, until February 25. Except for headaches and general aches in his legs, there were no symptoms accompanying the fever. The cough subsided after the first day. An x-ray of the chest taken on admission showed slight clouding of the left lower lobe but 3 subsequent films, including 1 taken on February 9, showed the lungs to be normal. No cause for the continued fever could be determined. Throat washings were taken for virus isolations a few hours after entry. All bacteriological studies were negative for pathogenic organisms in the sputum, stools, and urine, and serological studies for enteric agglutinins, cold hemagglutinins, and heterophile antibodies were all negative.

Case A-2. A 39-year-old Italian iron worker was admitted to the hospital on January 31 complaining of breathlessness, cough, and bloody sputum. He was well until January 23 when he had a slight "head cold" and then began to feel worn out and depressed with general malaise, anorexia, apathy, and a feeling of fullness in the head. This continued until January 26 when he had several shaking chills in rapid succession and these were followed by fever and cough. Two days later the cough increased and became productive of bloody sputum which he continued to raise until the time of admission to the hospital. At that time, the temperature was 98.4° F., pulse 100, respirations 28, and blood pressure 138/88. The patient was extremely dyspneic, cyanotic, and breathing with audible tracheal râles. He was raising copious amounts of sputum containing dark red blood. There was diffuse dulness of both lungs and loud bubbling râles were heard. X-ray showed diffusely scattered nodular consolidation throughout both lung fields. The white blood count was 19,000 with 88 per cent polymorphonuclear neutrophils. The patient died within a few hours. Autopsy showed a diffuse hemorrhagic bronchopneumonia which is described elsewhere (1). Some of the lung was used for virus isolation.

Case A-3. A 61-year-old painter was admitted in a moribund condition on March 6 and died a few hours later. From his relatives it was learned that he had felt unusually tired during the preceding 2 months. One week before entry, he had symptoms which might be interpreted as those of clinical influenza. This, however, did not keep him from work. On March 4, while at work, he had a severe shaking chill and, by the time he reached home, he felt markedly feverish and prostrated. On the next day, he had chilly sensations, pain in the right lower chest which was severe and pleuritic in character, and cough productive of large amounts of dark rusty sputum. These symptoms increased in severity.

On arriving at the hospital, the patient's temperature was 103° F., pulse 136, respirations 42, and blood pressure

158/78. He was markedly dyspneic and cyanotic, coughing and raising rusty sputum, grunting with obvious pleuritic pain. There were signs of frank consolidation of the right lower lobe and diffuse moist râles throughout the rest of the lung. The leukocyte count was 2000 of which 60 per cent were polymorphonuclear neutrophils, most of them immature. The sputum was loaded with type I pneumococci in pure culture, both by direct Neufeld test and by direct culture, and the same organism was cultured from the blood. In spite of oxygen, intravenous sulfonamides, antipneumococcic serum, and penicillin, the patient failed to register improvement and died 10 hours after entry. Autopsy showed lobar pneumonia of the right lower lobe, seropurulent effusion of the right pleura, and a hemorrhagic congestion of the rest of the lung. Some of this hemorrhagic lung was used for virus isolation. Type I pneumococcus was obtained from cultures of the heart's blood, all lobes of the lungs, and the pleural fluid. Details of the pathology are given elsewhere (1).

The results of the attempts to isolate virus in these 8 cases are summarized in the lower part of Table I and the bacteriological findings are listed on the right hand side of Table II. The viruses were isolated with about the same ease as in the epidemic cases. They were obtained from unfiltered and filtered materials, both in mice and in chick embryos. In Case 70, however, one attempt with unfiltered material and another with the filtered washings in eggs were abandoned as failures. The former was contaminated with hemolytic streptococci and caused early deaths of the embryos. Virus was obtained from the same unfiltered washings in mice.

The viruses isolated from the fatal cases gave typical serological reactions of influenza A when tested with immune rabbit serums. All the others gave atypical reactions although none of them reacted to a higher titer with anti-B than with anti-A serum. A study of these strains and their relationship to typical strains of A and B virus as well as to the epidemic strains, is now in progress.

In Table VI are shown the results of tests for influenzal antibodies in these 8 cases and in 3 other cases of clinical influenza which were observed at the same time but in which virus isolations were not attempted. The poor antibody response in this group of cases is in sharp contrast to the definite and sharp increase in influenza A antibodies in the epidemic cases shown in Table III. In only 1 case in the present group was there a 4-fold rise in titer against the homologous virus and none showed

TABLE VI

Influenzal antibodies in clinical cases of influenza occurring between January 23 and March 11, 1944

Case no.	Date of onset	Date of serum	Days after onset	Titers of influenzal antibodies					
				PR8(A)		Lee(B)		Homologous	
				C.F.	A.I.	C.F.	A.I.	C.F.	A.I.
66	Feb. 5	Feb. 8	2	<8	16	<4	8	8	128
		Feb. 16	10	<8	32	<4	4	8	128
		Feb. 28	22	8	16	<4	4	8	128
		Apr. 12	66	<8	16	<4	8	8	128
67	Feb. 7	Feb. 11	4	32	16		8	<8	<4
		Feb. 17	10	32	16		8	<8	<4
		Mar. 2	24	32	16		8	<8	4
		Mar. 22	44	32	16		4	<8	8
68	Feb. 26	Feb. 29	3	8	64	32	32	16	64
		Mar. 7	10	8	128	32	32	16	64
		Mar. 14	17	8	128	32	32	16	64
		Mar. 21	24	8	128	32	32	32	64
		Apr. 12	46	8	128	32	32	16	64
69	Mar. 5	Mar. 12	7	<8	16	<4	16	<8	8
		Mar. 18	13	<8	32	<4	16	<8	16
		Mar. 28	23	<8	32	<4	8	<8	16
70	Mar. 6	Mar. 8	2	<8	32	<4	8	<8	16
		Mar. 20	14	<8	32	<4	8	<8	16
		Apr. 12	37	8	32	<4	8	<8	16
71	Mar. 11	Mar. 12	1	<8	16	8	8	<8	8
		Mar. 27	16	<8	16	8	8	<8	16
72	Jan. 24	Jan. 31	7	8	<4		<4		
		Feb. 7	14	8	<4		<4		
73	Jan. 27	Feb. 8	12	<16	4		16		
		Feb. 15	19	16	4		16		
		Feb. 23	27	16	8		16		
		Mar. 7	40	16	8		32		
74	Feb. 23	Mar. 1	7	8	64		4		
		Mar. 8	14	8	64		4		
A2	Jan. 23	Feb. 1	9	16	8		4		4
A3	Mar. 4	Mar. 6	2(?)	64	64		256		256

Note: In Cases 67, 73, A2, and A3, the C.F. tests with PR8 were done 3 to 4 months before the corresponding A.I. tests. In all other instances, the 2 tests were done on the same sera, simultaneously. In Case 74, there was *Pn. 9* pneumonia, onset Feb. 28. In Cases A2 and A3, viruses were isolated from the lungs at autopsy. The sera in these 2 cases were obtained on the day of death.

more than a 2-fold rise in titer of PR8 antibodies. Furthermore, a titer of 64 or higher with any of the viruses was obtained in only 4 cases, including 1 that was fatal. The others showed no significant titers or rises in titer with their own, the PR8, or the Lee strains.

RESULTS OF TESTS FOR INFLUENZA B ANTIBODIES

Tests for influenza B antibodies were done by the agglutination-inhibition method in almost all

of the sera. All of the acute and convalescent sera from the epidemic and post-epidemic cases were included. The Lee strain of influenza B was used in these tests. The results are summarized in Table VII according to the various groups of cases. Maximum titers of 64 or higher were obtained in only 5 cases, as follows:

Case 21. (Table IV) Influenza began December 20. Serum of January 21 tested with PR8 showed titers of 128 and 64 by complement fixation and agglutination

TABLE VII

Results of Hirst inhibition tests with the Lee strain of influenza B in 6 groups of cases

Group	Listed in table	Maximum titer						Total
		<8	8	16	32	64	128+	
I	III	2	1	6	4			13
II	IV	3	2	3	3	1		12
III	V	2	5	10	4			21
IV	##	2	3	6	3	1		15
V	##	1	8	10	5	1	1	26
VI	VI	3	4	1	2		1*	11
Total		13	23	36	21	3	2	98

* Titer 256.

These tables omitted to conserve space. Group IV includes the persons who gave a history of simple respiratory infections and Group V includes those who denied having any upper respiratory tract infections.

inhibition, respectively. The inhibition titer with the Lee strain was 64.

Case 51. Common cold (?) began December 26 and pneumococcus type 7 pneumonia began January 29 and was complicated by pneumococcal endocarditis. The sera of February 19 and 28 had titers of 64 and 32, respectively, of inhibition with the Lee strain. The titer against PR8 in both sera was 16.

Cases 85 and 97. The titers with the Lee strain in sera of January 22 and February 9, were 64 and 128, respectively. The former was from a case of peptic ulcer and the latter from a case of lung abscess. Both had titers of 16 or less against PR8. There was no history of recent upper respiratory tract infection in either case.

Case A-3. (Table VI) In this case, the serum taken shortly before the patient died had a titer of 64 when tested with PR8 and with the homologous strain isolated from the lung. The titer against the Lee strain was 256.

In none of these cases was a significant rise in titer demonstrated. In Case 11, however, there was a 4-fold rise during convalescence from an original level of 8. This was one of the epidemic cases and the same sera showed a rise in titer against PR8 from 16 to 256 by the complement fixation test and from 32 to 128 by the inhibition test.

It is apparent, therefore, that significant titers of influenza B antibodies were uncommon and some of the few that were encountered were associated with high titers of influenza A antibodies. Significant rises in titer to influenza B alone were not demonstrated in any case. Indeed, the marked rises in titers against influenza A were accompanied by a slight rise in titer against the B strain in only 1 case.

DISCUSSION

The comparative ease with which isolation of virus was accomplished, both in the clinical cases and from the autopsy material during and after the height of the epidemic, is worthy of note. Two methods were used which in this laboratory have proved the simplest and most successful. The first is the intranasal inoculation of white mice and serial passage of lung suspensions. The second is the intra-allantoic inoculation of chick embryos followed by similar passage of the allantoic fluid. Both filtered and unfiltered materials were used in the first instance and yielded similar results. Filtration of lung suspensions or allantoic fluid was frequently necessary before subsequent passages when bacterial contaminants were encountered but these did not usually affect the virus content materially. In some of the later attempts, the initial inoculations of infected throat washings, sputum, or lung suspensions were immediately preceded by the injection of 2.5 to 5.0 mgm. of sodium sulfadiazine and 250 units of penicillin contained in 0.1 ml. of distilled water, intra-abdominally in mice and into the allantoic sac of the chick embryos. This permitted successful isolations from heavily infected materials such as sputum and lungs from cases of pneumonia. Similar results in this epidemic have been obtained with throat washings in chick embryos (16).

At first it was felt that it was essential to obtain materials for virus isolation during the first 2 days of illness and the 4 epidemic cases were so chosen. In later cases, however, isolations were accomplished from washings obtained on the third to the seventh day. This was considered possible after virus had been isolated from the lung in Case A-2, in which death occurred on the ninth day after the onset of the influenza. In a previous sporadic case, influenza A virus was isolated from the lungs of a patient who died on the seventh day (1). In the present study, the determining factor in the choice of clinical cases for virus isolation was that the patients were still acutely ill with typical symptoms of influenza and had a temperature of 102° F. or higher at the time when the material was collected. On the whole, the patients from whom viruses were isolated represented cases of

more than average severity and a large proportion of them had pulmonary complications.

Isolations of influenza A from the lungs of cases dying of staphylococcal pneumonia complicating influenza have been previously reported in one case (17) and in 3 cases (18). Presumptive evidence of the presence of influenza A virus was obtained (3) from the lung in a case of mixed staphylococcal and streptococcal pneumonia which occurred at the Boston City Hospital during the 1940-41 epidemic (19). During March and April 1943, influenza A virus was isolated from the lungs of 2 sporadic cases as already mentioned. One of them was a case of staphylococcal pneumonia, and no significant bacterial pathogen was obtained from the second case (1, 2). During the present study, influenza virus was isolated from the lungs in 3 fatal cases of pneumonia, one infected with *Staphylococcus aureus* and beta hemolytic streptococci, a second with type I pneumococcus, and a third in which no definite pathogen was found and only a few alpha hemolytic streptococci were obtained in cultures of the sputum and the lung.

The identity of most of the viruses isolated from cases during the height of the epidemic was readily established as influenza A both by neutralization tests in mice and by agglutination-inhibition tests with influenza A (PR8) and B (Lee) antisera. The reactions of these viruses closely resembled that of PR8 except those from Case 4 and Case A-1 which reacted to a considerable, though lesser degree with the influenza B antiserum. The antibody response of the epidemic cases was, on the whole, quite characteristic of infection with a strain resembling PR8. In the 4 clinical cases from which virus was isolated, furthermore, the antibody response to PR8 in each instance closely paralleled that obtained against the homologous strain of virus. These findings, though not very extensive, suggest that the agent responsible for most of the cases during the height of the epidemic, at least the severe ones among them, was an influenza A virus resembling PR8.

In the post-epidemic cases, on the other hand, both the viruses isolated and the antibody responses observed were quite different. Sharp increases in antibody titers against PR8 were not encountered. Indeed, only minor rises in

titer, or none at all, were demonstrated, even against the homologous viruses. Some of the latter, although they reacted with PR8 antiserum in moderate or high titer, also reacted with the type B (Lee) antiserum to the same degree or only slightly less. Wide antigenic differences among strains of influenza A have also been noted by several investigators (20 to 22) and among strains of influenza B by another (23). In the present study, the fact that the atypical viruses and antibody responses were obtained, for the most part, some time after the height of the epidemic, suggests the possibility that the antigenic properties of the epidemic strain may have been modified, either by repeated passage in non-susceptible individuals or by prolonged residence in the hosts before manifest infection took place. Alternatively, it could be assumed that these viruses were merely latent ones which the patients were harboring as carriers and which had no relation either to their disease or to the epidemic, but this explanation seems less likely in view of the characteristic symptoms in these cases. The antigenic properties of both groups of viruses and their relationship to each other and to other influenza viruses is now under investigation in collaboration with Dr. John F. Enders and Miss Elizabeth G. Mills.

The isolation of influenza viruses from the lungs of fatal cases of bacterial pneumonia serves also to emphasize the significance of the finding of influenzal antibodies in high titers in other severe cases of pneumonia, observed during and after the height of the epidemic. Similar though less definite findings were reported (3) in the serum of cases of pneumococcal and staphylococcal pneumonia occurring at this hospital during the influenza epidemic of 1940-41. The possible relation of the influenza A virus to the pulmonary infections was suggested at that time and this suggestion is fully supported by the present findings. As in the previous epidemic (24), an unusual number of cases of *Staphylococcus aureus* pneumonia again occurred as complications of the influenza and some of them were fulminating in character. The rarity with which hemolytic streptococcal complications occurred in the last 2 epidemics is in sharp contrast to their well-known prevalence in the pandemic of 1918.

High antibody titers for influenza A were obtained in the patients with pneumonia and in the other persons who gave a history of the symptoms of influenza during the epidemic. These are in sharp contrast to the low titers observed at the same time in persons who had symptoms of a common cold or of tonsillitis and in those who denied having had any symptoms of upper respiratory tract infection during the epidemic. This stands out clearly in the summary of the maximum titers of influenza A antibodies which were obtained in the different groups of cases, as shown in Table VIII. To be sure, there were individual instances of high titers among the persons without influenza and of comparatively low titers in some of those who had symptoms of the disease, but these were few. The contrast between these groups of cases appears to be significant and the combinations of the high titers and the symptoms probably represents the result of influenzal virus infection during the epidemic. The findings also suggest that other simple respiratory infections may occur during an epidemic of influenza and many of them probably can be differentiated from the influenza cases both clinically and by their failure to elicit the proper antibody response. It is of interest that antibody titers in the post-epidemic cases of influenza when viewed as a group in this perspective, correspond more closely to those found in the non-influenza groups of cases, again emphasizing the atypical character of these post-epidemic cases.

It has been noted, in connection with each of the groups of cases, that the titers of PR8 antibodies obtained in the individual serums by

both the complement fixation and by the agglutination-inhibition tests were in close agreement. Most of the differences that were noted indicated a 2-fold higher titer with the former. These findings are in essential agreement with those of another worker (20) when account is taken of the different methods of expressing the titers used by this author for the 2 tests. In reviewing the present data, and after repeating a number of the tests, it became evident that most of the large discrepancies could be accounted for by the deterioration of the influenzal antibodies in the sera on storage at refrigerator temperatures (5 to 10° C.), and, in addition, contamination may have accounted for some of them. Except in some of the post-epidemic cases listed in Table VIII, the complement fixation titers were all carried out within 2 months of the time when the sera were collected. The agglutination-inhibition titers were done from 3 to 5 months later. The validity of the complement fixation titers is attested by the fact that a simultaneous control serum preserved at -72° C. retained its high titer throughout and a number of the sera showed the same titers when retested within the first 2 months.

A number of the sera were later retested with the same preparation of PR8 virus and both tests run simultaneously. This time most of the sera showed lower complement fixing titers than in the tests done 5 months previously and many of them gave 8- or even 16-fold titers by the agglutination-inhibition test. In a few sera, however, the titers were the same with both tests. The titers of agglutination-inhibition obtained in the later tests of these sera were

TABLE VIII

Maximum titers of antibodies for influenza A (PR8) in 6 groups of cases

Group*	Complement fixation										Agglutination—Inhibition									
	<16	16	32	64	128	256	512	1024	2048	Total	<16	16	32	64	128	256	512	1024	Total	
I			1	1	2	3	2	2	2	13				2	2	5	4		13	
II	1	1		4	6		1			13	2	1	2	7		1			13	
III			2	4	8	5	4	1		24			2	10	5	2	3	2	24	
IV	6	3	6							15	6	7							15	
V	11	3	7	2	2					25	15	4	3	4					26	
VI	7	2	1	1						11	3	2	3	2	1				11	
Total	25	9	17	12	18	8	7	3	2	101	26	14	12	25	8	8	7	2	102	

* See Table 7.

essentially the same as those found at the time of the previous tests which were done 1 to 2 months previously. Discrepancies, however, were noted occasionally with this test when different preparations of virus were used as previously observed by others (25).

From these observations, it was inferred that the antibody titers probably had deteriorated on storage, and, in a few instances, because of contaminations. The findings also suggested that the rate at which the deterioration took place varied markedly in the individual sera. Possibly also, the 2 antibodies deteriorated in some sera at different rates, but this aspect requires further study. The absolute values shown for the agglutination-inhibition titers may, therefore, not represent the actual titers originally present in those sera. This, however, does not detract from the value of the observations made since most of the inhibition tests were carried out within a brief period. Furthermore, the results of these tests corresponded quite closely with those obtained by the complement fixation tests which were carried out some time previously. The findings, however, suggest that for the most reliable results and comparisons, tests for influenzal antibodies should be carried out within a short time after the sera are collected. Special care is probably required for the preservation of the sera in order to avoid deterioration of these antibodies.

SUMMARY AND CONCLUSIONS

Influenza viruses were isolated early in the course of the disease from throat washings and also from the lungs of fatal cases of pneumonia, both during and after the height of the epidemic of influenza which occurred in Boston in December 1943 and early in January 1944. Successful isolations were made from filtered and unfiltered materials, both by the intranasal inoculation of mice and by allantoic inoculations in chick embryos.

The reactions of the viruses obtained during the height of the epidemic and the antibody response to characteristic infections occurring at that time indicated that the epidemic cases were caused chiefly by strains of influenza A similar to PR8.

The post-epidemic cases were atypical, not only with respect to the viruses which were isolated from some of them but particularly in their antibody responses, both to their own strains and to the standard PR8 strain.

No definite evidence of infection with influenza B was obtained in any of the cases studied, either during or after the epidemic.

In the sera of persons who gave a characteristic history of influenza during the epidemic, significant titers of antibodies to the PR8 strain of influenza A were demonstrated for as long as 12 weeks after the onset of these symptoms. Such significant titers were not demonstrated during the same period in cases of pneumonia nor in other persons who either denied having any symptoms of acute respiratory infection or had symptoms which could be recognized as those of the common cold and not influenza.

These findings suggest that characteristic clinical histories and serological findings obtained as long as 2 or 3 months after a definite epidemic of clinical influenza may serve to differentiate most, but not all of those who, during the height of the epidemic, were infected with influenza virus from those who had other types of respiratory infections. The same does not hold true for acute respiratory infections which occur after the epidemic has subsided.

Of particular interest were the high titers of antibodies to PR8 that were demonstrated in severe cases of bacterial pneumonia which occurred during and shortly after the epidemic and in which there was a recent history of clinical influenza. These findings and the isolation of influenza virus from the lungs in 3 fatal cases suggest that the occurrence and severity of the pneumonia in such cases is related to the antecedent infections with the influenza virus.

The most frequent organisms obtained in the pneumonias which followed influenza were pneumococci of various types and *Staphylococcus aureus*. No significant bacterial pathogen could be recognized in some of the pneumonia cases, including a fatal one in which a virus was recovered from the lung. Both *Staphylococcus aureus* and hemolytic streptococcus were obtained from one of the other fatal cases and type 1 pneumococcus was cultured from the third.

Comparable results were obtained in this study with the complement fixation and agglutination-inhibition tests.

Evidence was also obtained that influenzal antibodies may deteriorate after a few months in sera stored at 5 to 10° C.

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STUDIES ON PRIMARY ATYPICAL PNEUMONIA.

I. CLINICAL FEATURES AND RESULTS OF LABORATORY INVESTIGATIONS^{1, 2}

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"Primary atypical pneumonia, etiology unknown" (1) is one of many terms used to designate an acute respiratory infection which has been widely prevalent in recent years. The illness is characteristically gradual in onset, with constitutional as well as respiratory symptoms, and pulmonary changes more manifest in roentgenograms than by physical examination. The course of illness varies considerably in duration and severity. Complications are uncommon and although convalescence is frequently protracted the illness almost invariably terminates with complete recovery. Therapy with sulfonamide drugs is not effective.

Contemporary interest in this form of pneumonia was aroused by several fairly recent reports (2 to 4), although one author (5) had observed patients with a similar illness several years earlier. Numerous other descriptions and investigations of primary atypical pneumonia have since been published, and have been reviewed recently (6 to 9). In some of these reports, unusual outbreaks of acute respiratory illnesses described during the past century were cited to show that primary atypical pneumonia is probably not a new disease. Nevertheless, it is apparent from these reports that, in recent years, the incidence of the disease has actually increased. The rate of its occurrence in the civilian population is not known. In the armed

forces, however, it has appeared to be more prevalent than the pneumococcal pneumonias and the total number of man days lost as a result has been considerable.

The term "primary atypical pneumonia" has been useful in distinguishing the syndrome under discussion from clinically similar illnesses due to known infectious agents (10) such as *Rickettsia diaporica* (11) and members of the psittacosis group of viruses (12, 13).

Although primary atypical pneumonia has been frequently referred to as "virus pneumonia" and most investigators have considered it to be of non-bacterial origin, the causative agent, or agents, in the majority of cases has remained obscure. The recovery of a number of different viruses (14 to 18) from certain patients with primary atypical pneumonia has been reported but the importance of any of these agents in the pathogenesis of the disease has not been established.

An investigation of primary atypical pneumonia has been carried out continuously at the Hospital of The Rockefeller Institute since February, 1942. Because of circumstances which afforded extensive clinical as well as laboratory facilities, it was possible while investigating the pathogenesis of primary atypical pneumonia to conduct also an appraisal of its clinical aspects. An effort was made to correlate these two lines of investigation and to study each patient in as comprehensive a manner as possible.

Recently, a non-hemolytic streptococcus, isolated from the lungs of 2 patients who died of primary atypical pneumonia, was found to react with sera obtained, during convalescence, from other patients with this illness but did not react with sera obtained during the acute stage of the disease (19). For convenience in reference, this

¹ The Bureau of Medicine and Surgery does not necessarily undertake to endorse the views or opinions which are expressed in this paper.

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microorganism has been designated streptococcus MG. Because of the possibility that streptococcus MG might have more than an incidental association with primary atypical pneumonia, the properties of this microorganism and its relation to the disease were investigated. The bacteriological and serological characteristics of streptococcus MG are described in separate communications (20, 21). The special techniques for isolating streptococcus MG from the respiratory tract of human beings and an extension of the original observations concerning the relation of this microorganism to primary atypical pneumonia are described in the accompanying paper (22).

It is the purpose of the present paper to describe the clinical features of primary atypical pneumonia as observed in patients studied in the Hospital of The Rockefeller Institute, to present the results of various investigations pertaining to the disease, and to correlate some of these findings with the studies concerning streptococcus MG which have been referred to above (20 to 22).

From August 1, 1942, to March 2, 1944, 180 patients were admitted to this hospital with acute respiratory infections suspected to be primary atypical pneumonia. In each case, a careful and detailed record was made of the clinical features. Roentgenograms of the chest were made at frequent intervals and numerous clinical laboratory studies were carried out. In addition, samples of serum were obtained repeatedly throughout the illness and were stored at 4° C. for use in serological tests. Specimens of plasma, sputum, and nasopharyngeal washings, as well as specimens of pleural or cerebrospinal fluid, were examined immediately after they were obtained and portions of each specimen were preserved at -70° C. for use in subsequent investigations.

From the completed records of these 180 patients, 106 were selected on the basis of clinical and laboratory criteria as representative examples of primary atypical pneumonia. The criteria used for selection permitted the inclusion of only those patients who showed each of the following findings: (1) acute febrile respiratory disease; (2) pulmonary consolidation demonstrable by x-ray; and (3) adequate clinical and

laboratory evidence that the pneumonia was not due to pneumococcus or other microorganisms of recognized pathogenicity.

Ninety-five of the patients were male and 11 were female. All patients were young adults. These patients came from widely separated areas and had been exposed to multiple and diverse sources of infection. Nevertheless, as a group, they exhibited a remarkable degree of clinical homogeneity.

CLINICAL FEATURES

The clinical features of primary atypical pneumonia, as observed in these 106 patients, were on the whole similar to those which have been described in other reports. The onset of illness was gradual in 73 per cent of the patients and, in contrast to lobar pneumonia, was usually ill-defined. Constitutional symptoms generally preceded those referable to the respiratory tract. In many instances, the initial complaint, variously described by the patient, suggested a combination of lassitude, weakness, and fatigue.

TABLE I
Symptoms in 106 patients with primary atypical pneumonia

	Symptoms	Patients	
		Number	Per cent
General	Headache	69	65
	Malaise	65	61
	Chills or chilliness	63	59
	Generalized aches	30	28
	Anorexia	37	35
	Nausea	30	28
	Vomiting	25	24
Respiratory	Cough	104	98
	Sputum	87	82
	Bloody sputum	27	25
	Sore throat	32	30
	Chest pain		
	Substernal	25	24
	Lateral	16	15
	Epistaxis	16	15
	Dyspnoea	5	5

In Table I, the commoner symptoms have been listed, together with the frequency of their occurrence. Chills, fever, headache, and cough developed early and occurred frequently. Shaking chills were rare but sensations of chilliness, sometimes recurring, were experienced by 59 per cent of the patients. Headache, usually generalized but sometimes predominantly frontal, occasionally associated with photophobia, and ranging from mild to extremely severe, oc-

curred in 65 per cent of the patients. Aching of the trunk and extremities was present in only one-fourth of the patients and as a rule was mild. About a third of the patients exhibited an appreciable degree of anorexia and nausea. Vomiting occurred at some stage of illness in one-fourth of the patients but this symptom was never a conspicuous or severe manifestation of the disease.

Cough, one of the most constant and characteristic features of primary atypical pneumonia, was present in all but 2 of the 106 patients. In some instances, this was the first symptom to appear; in others, it developed gradually after a lapse of several days. Frequently, it became paroxysmal and exhausting. Coughing, although dry at first, was ultimately productive of sputum in 82 per cent of the patients. The sputum of one-fourth of the patients contained blood, usually, however, only as streaks or traces. Grossly bloody or "rusty" sputum was uncommon. Sore throat when present was rarely severe, and, in many instances, appeared to result from the irritation of coughing.

In association with the cough, about one-fourth of the patients experienced dull, burning, or oppressive substernal discomfort. Less frequently, sharper pains developed which were referred to the thorax laterally.

Epistaxis occurred in 16 patients and syncope in 3. Several patients complained of transient numbness and tingling of the extremities. Abdominal pain was only an occasional and then unimpressive complaint.

The results of physical examinations of these patients are summarized in Table II. On admission, most of the patients did not appear to be very ill. An elevation of temperature was noted during hospitalization in all but 5 instances. Cyanosis was rarely present, and herpetic lesions were observed in only 3 instances. Respirations were usually normal in rate and seldom labored. Tachypnea was seen in only 14 of the more severely ill patients. Relative bradycardia, however, observed in over two-thirds of the patients, was a frequent finding during the early febrile phase of illness, and a feature of considerable diagnostic assistance in distinguishing clinically between this syndrome and pneumococcal pneumonia. Signifi-

TABLE II

Abnormal physical signs in 106 patients with primary atypical pneumonia

Physical signs	Patients	
	Number	Per cent
Fever	101	95
Nasal congestion	60	57
Pharyngitis	73	69
Cervical adenopathy	25	24
Pulmonary signs	104	99
Dulness	57	54
Altered breath sounds	74	70
Râles	99	93
Friction rub	7	7
Fluid	1	1
Bradycardia	72	68
Tachycardia	8	8
Tachypnoea	14	13
Cyanosis	12	11
Signs referable to the central nervous system	6	6
Palpable spleen	1	1

cant tachycardia was infrequent and, when present, usually developed during convalescence.

Nasal congestion and mild pharyngitis were noted respectively in 57 and 69 per cent of the patients. Cervical lymph nodes were enlarged in one-fourth of the patients, but usually were not prominent or tender.

Physical signs of pulmonary disease, although much less definite than in lobar pneumonia, were nevertheless detected at some stage of illness in all but 2 of the 106 patients. Dulness to percussion, usually of slight degree, was noted over the affected area in more than half of the patients. Alterations in the breath sounds, usually harshness or diminished transmission, occurred in 70 per cent of the patients. On the other hand, breath sounds of tubular quality and pectoriloquy were rarely heard. Râles, usually fine and subcrepitant during the early stages of illness, later coarse and more moist, were detected in 93 per cent of the patients. Often râles could be heard only after coughing or at the end of deep inspiration. A pleural friction rub was heard in 7 instances and, in 1 patient, signs of pleural effusion were detected. A few patients with severe headache had slight stiffness of the neck. One patient briefly lost the power of ocular accommodation. The spleen was palpable in only 1 instance. Abdominal distension, so common in lobar pneumonia, was conspicuously absent. No other relevant abnormalities were noted on physical examination.

The clinical course of illness in the present

group of patients was extremely variable. All recovered, but it was not possible to predict accurately either the duration or the severity of illness in any given case. In the total group of patients, elevations of temperature to 100° F. or more were present for an average of 10.1 days, ranging in individual patients from 0 to 41 days. Physical signs of pneumonia were present for an average of 12.8 days, but also ranged from 0 to 41 days. The maximum recorded temperature averaged 103.5° F. with a range of 99 to 106° F. The charted temperature curves of individual patients also showed great variation. The commonest type of curve was moderately remittent, and rarely was either high persistent fever or markedly intermittent fever seen. Defervescence was usually by lysis over a period of more than one day. Hospitalization was required for an average of 33 days with a range of 10 to 73 days.

Complications were rare and usually not of great significance. Pleuritis occurred in 6 patients. In only 1, however, was the accumulation of fluid sufficient to warrant thoracentesis. In the others, the amount of fluid was evidently small and, in several, it was located principally in the interior fissures. Acute sinusitis developed in 8 patients and non-suppurative otitis media occurred twice. One patient had severe stomatitis and gingivitis. Another patient, during convalescence from primary atypical pneumonia, contracted lobar pneumonia due to pneumococcus Type XIV, evidently by contact, and in both infections, the left lower lobe was the site of consolidation.

Although no systematic investigation of the therapeutic value of sulfonamides in primary atypical pneumonia was carried out, 32 patients in this study received sulfathiazole or sulfadiazine during the course of illness. Of these, 16 received an adequate therapeutic trial over a period of 4 to 9 days, with total dosages of 21 to 50 grams. Ten patients showed no evidence of a therapeutic response. In 6 patients, the temperature returned to normal within 72 hours of the initiation of treatment, but in all 6, defervescence occurred between the sixth and tenth days of illness. Moreover, in 2 of these patients, the fall in temperature occurred within 24 hours, before an adequate dose had been given and

before a response could reasonably be expected. These findings are in agreement with previous observations that sulfonamides do not obviously benefit patients with this disease.

X-RAY FINDINGS

X-ray films of the lungs, taken at frequent intervals throughout the course of illness, showed evidence of more extensive pulmonary lesions than the findings on physical examination would have led one to expect. Data concerning the site, extent, and duration of pulmonary consolidation as determined by multiple serial roentgenograms on each patient are presented in Table III. In this study, the word "con-

TABLE III

Distribution, extent, and duration of pulmonary consolidation as determined by serial x-ray films in 106 patients with primary atypical pneumonia

	Number	Patients	Per cent
Lobe involved			
Left lower	66 (18)*		62.2 (16.9)
Left upper	19 (2)		17.9 (1.9)
Right lower	64 (20)		60.3 (18.9)
Right middle	22 (3)		20.7 (2.8)
Right upper	25 (7)		23.5 (6.6)
Number of lobes involved			
1	50		47.1
2	38		35.8
3	8		7.5
4	4		3.8
5	6		5.7
Duration of consolidation			
1 week	9		8.4
2 weeks	51		48.1
3 weeks	25		23.4
4 weeks	11		10.3
5 weeks	8		7.5
6 weeks	2		1.9
7 weeks	0		

* Numbers in parentheses indicate patients in whom the designated lobe was the only site of pulmonary consolidation.

solidation" has been used in a broad sense to designate findings thought to represent pneumonic involvement of the lung parenchyma. In referring to x-ray films of the lung, this term is used irrespective of the extent or density of the pneumonic shadow.

As shown in Table III, the site of pulmonary consolidation was most frequently in the lower lobes and the incidence of involvement was almost identical on the 2 sides. Consolidation was present in the left and right lower lobes in 62.2 and 60.3 per cent of patients, respectively. Each of these lobes was the only site of consolida-

tion, however, in 16.9 and 18.9 per cent of patients, respectively. Consolidation was present in the left and right upper lobes and the right middle lobe in 17.9, 23.5, and 20.7 per cent of patients, respectively. On the other hand, each of these lobes was the only site of consolidation in 1.9, 6.6, and 2.8 per cent of patients, respectively.

Pulmonary consolidation as observed in roentgenograms was confined to a single lobe in 50 patients, located in one of the lower lobes in 38 patients, in one of the upper lobes in 9 patients, and in the middle lobe in 3 patients. Pulmonary consolidation occurred in more than one lobe in 56 patients. Two lobes were involved in 38 patients, 3 lobes in 8 patients, 4 lobes in 4 patients, and all 5 lobes in 6 patients. The average number of lobes involved for all patients was 1.85.

In some instances, multiple lobes were involved simultaneously, in others consolidation extended from one lobe to another or new shadows appeared in one or more lobes during the course of illness. Occasionally, fresh areas of consolidation appeared while other areas were undergoing resolution, and consolidation was observed to recur in the same site or to reappear in another area after the initial lesion had cleared. Thickening of the interlobar septa, occasional small collections of fluid in the interlobar fissures or in the costophrenic angles, and, in films of one patient, definite pleural effusion were also observed.

The individual pulmonary lesions as viewed in roentgenograms showed extraordinary variation in density as well as in distribution. Usually, an area of pneumonic consolidation was present in the initial film of the chest taken at the time of admission, even as early as the first or second day of illness. In a few patients, evidence of pneumonia was not present on admission but appeared subsequently. In some of these patients, consolidation first appeared as an isolated area well out in the lung parenchyma without obvious connection with the hilar shadow. In most instances, however, the initial consolidation made its appearance and was most dense at the hilum, spreading and becoming less dense toward the periphery of the lung. The borders of the consolidated areas were usually irregular,

ill-defined, and not always confined by interlobar boundaries. Sometimes the shadow was present only as a diffuse haze; in other instances, it was quite dense. Only rarely, however, was the density or extent of consolidation in a given lobe comparable to that seen in lobar pneumonia. In other instances, consolidation was mottled and sometimes the appearance of small abscess cavities was suggested. Infiltrations radiating from the hilum and involvement of the upper lobe resembled tuberculosis even more frequently, and other lesions, especially in the lower lobes, simulated bronchiectasis. The relative rapidity with which the lesions cleared in primary atypical pneumonia assisted in excluding a diagnosis of chronic pulmonary disease. Other forms of pneumonia could not be distinguished from primary atypical pneumonia in a given case on the basis of roentgenograms alone.

Resolution of the consolidated areas was observed to occur in several ways. In most instances, clearing proceeded from the periphery of the lung toward the hilum but occasionally resolution progressed in the reverse direction. Clearing was also observed to occur by progressive reduction in the size of the affected area and by a diminution of density until no evidence of consolidation remained. In many instances, during the process of resolution, the affected area assumed a diffusely mottled appearance. Frequently, after all parenchymal involvement was thought to have disappeared, the bronchovascular markings remained prominent.

In this group of patients, the duration of consolidation in roentgenograms was arbitrarily calculated as the interval from the onset of illness to the last day on which evidence of parenchymal involvement was still visible. As recorded in Table III, pulmonary consolidation had disappeared by the end of the second week of illness in more than half the patients and by the end of the third week in 80 per cent of the total number. The average duration of consolidation as determined by serial roentgenograms was 14.2 days with a range of 3 to 41 days.

Although in the present study, roentgenological evidence of pulmonary consolidation was established as one of the essential criteria for a diagnosis of primary atypical pneumonia, it

should be emphasized that the x-ray shadows observed were not distinctive of this disease syndrome exclusively and a diagnosis of primary atypical pneumonia could not be made on the basis of roentgenological evidence except in conjunction with clinical and laboratory findings.

CLINICAL LABORATORY FINDINGS

The total leukocyte count on admission and at various intervals thereafter was usually normal or only slightly elevated. This was another helpful feature in distinguishing primary atypical pneumonia from pneumonia due to pneumococci or other bacterial agents in which leukocytosis characteristically occurs. Data concerning the total leukocyte counts in the present group of patients are summarized in Table IV.

The total leukocyte counts recorded upon the 106 patients with primary atypical pneumonia during each week of illness were grouped in 4 categories on the basis of their magnitude. These included total counts of less than 5000, those of 5000 to 10,000 considered to represent the normal range, those from 10,000 to 15,000, and those of more than 15,000.

It is apparent that throughout the course of illness as measured in weeks, considerably more than half the total leukocyte counts were within the normal range, and two-thirds or more of the counts were less than 10,000. Leukopenia was relatively infrequent and never extreme. The lowest recorded count of 3300 was observed in the second week of illness. Total leukocyte counts above normal were in most instances between 10,000 and 15,000. Counts of more

than 15,000 occurred during the first 3 weeks of illness but were uncommon. The highest count noted was 25,800 and occurred in the second week of illness. Sudden and unexplained shifts in the total counts sometimes occurred. Usually, however, in individual patients, neither high nor low counts were sustained on repeated examinations. The differential leukocyte pattern as observed in these patients was within normal limits.

The erythrocyte sedimentation rate was almost always increased during the acute phase of illness, and the accelerated rate usually persisted for some weeks during convalescence. A 5.0 ml. specimen of blood was collected in a tube containing dried potassium and ammonium oxylate and, within an hour, was transferred to a calibrated 100 × 3 mm. tube for the test. Readings were made at the end of 1 hour without correction for hematocrit, inasmuch as significant anemia was rarely present. The maximum rate was less than 10 mm. per hour in 4 per cent of patients, 10 to 19 in 2 per cent, 20 to 29 in 13 per cent, 30 to 39 in 22 per cent, 40 to 49 in 49 per cent, 50 to 59 in 9 per cent, and more than 60 in 1 per cent. A sedimentation rate of more than 15 mm. in 1 hour persisted on the average for 28.5 days with a range of 9 to 73 days. In a few instances, the sedimentation rate remained or became normal while the patient was still acutely ill. This observation was amply confirmed but not satisfactorily explained.

The urine was usually normal. Slight or moderate albuminuria was noted during the acute phase of illness in 22 per cent of the pa-

TABLE IV

Total leukocyte counts in 106 patients with primary atypical pneumonia

Total leukocyte count	Percentage of leukocyte counts* per week of illness									
	Week of illness									
	1	2	3	4	5	6	7	8	9	10
<5,000	5.9	8.0	1.9	9.7	5.9	13.3				
5,000 to 10,000	66.7	57.3	66.0	74.2	88.2	80.0	77.0	50.0	100.0	100.0
10,000 to 15,000	26.2	29.3	22.6	16.1	5.9	6.7	23.0	50.0		
>15,000	1.2	5.3	9.4							
Number of patients	84	75	53	31	17	15	13	4	1	2

* Only one count per patient per week was included in this analysis.

tients. Examination of the urinary sediment revealed granular or hyaline casts and transient occult hematuria in 10 and 8 per cent of the patients, respectively. Three of the 9 patients with microscopic hematuria had previously received sulfonamide therapy.

Lumbar puncture was done only when severe headache and meningismus were present. Specimens of cerebrospinal fluid from 6 patients were examined. Two contained lymphocytes (less than 50 per c. mm.) and gave weakly positive tests for protein. The other 4 fluids were normal.

ELECTROCARDIOGRAMS

Serial electrocardiograms⁵ were taken on 50 of the patients with primary atypical pneumonia, none of whom gave a history of previous cardiovascular disease. No significant deviations from normal were detected during any stage of the illness from the third through the thirtieth day after onset. Specifically, there was no increase in the P-R interval, no elevation or depression of the S-T segment, and no T-wave change.

BIOCHEMICAL STUDIES

Biochemical assays⁶ were carried out upon specimens of blood and urine from a number of representative patients. It was found that the icteric indices, prothrombin, serum CO₂, serum chloride, and plasma protein levels were within normal limits. Plasma carotene and vitamin A levels were reduced but only to a degree commonly encountered in other acute infectious diseases. As previously reported (23), chloride balance studies indicated that there was no striking disturbance of chloride metabolism, and plasma α -amino acid levels were found to be within the normal range. These latter two findings serve additionally to distinguish this syndrome from pneumococcal pneumonia, in which chloride metabolism is usually disturbed and the plasma α -amino acid levels are significantly reduced (24).

⁵Lt. C. G. Neumann (MC), U.S.N.R., kindly analyzed the electrocardiograms.

⁶Lt. Cdr. K. Emerson, Jr. (MC), U.S.N.R., Dr. C. L. Hoagland, Lt. Cdr. R. A. Phillips (MC), U.S.N.R., and Lt. Cdr. R. E. Shank (MC), U.S.N.R., kindly performed these analyses.

BACTERIOLOGICAL FINDINGS

The bacteriological flora of the nasopharynx and sputum was studied in each patient and an effort was made to obtain both qualitative and quantitative data. A culture of the blood was obtained from every patient at the time of admission and at intervals thereafter when indicated.

The cultures of the nose and throat in these patients yielded a variety of microorganisms. In cultures from occasional patients, one or another bacterial species appeared to predominate; but in the group as a whole, the bacterial flora appeared to be quantitatively and qualitatively similar to that commonly found in the upper respiratory passages of normal persons. The sputum of these patients also contained a variety of microorganisms, but, by ordinary methods of culture, no one species was found to be conspicuously or consistently present in specimens from the total group.

When pneumococci, streptococci, or *H. influenzae* were isolated from any source, an attempt was made to obtain specific identification by serological methods. The results are summarized in Table V.

Pneumococci were isolated from 54 per cent of the patients but in only 7 per cent was it possible to identify the organisms in the sputum directly by the Neufeld quellung technique. In most instances, pneumococci were isolated and identified following the inoculation of sputum into mice. Twenty-six different types of pneumococci were obtained from 57 of the 106 patients. It is noteworthy that pneumococci of types I and II, those most commonly associated with lobar pneumonia, were not found in any of the patients in this group. The distribution of pneumococcal types, as well as the number of pneumococci present in specimens from individual patients, was similar to the distribution of these organisms in the upper respiratory passages of normal persons.

Beta hemolytic streptococci were isolated from 10 patients, each of whom gave a history of recent contact with cases of streptococcal infection. These streptococci belonged to at least 9 distinct serological varieties. Five different types and 2 strains of as yet unidentified type

TABLE V

Bacteria isolated from 106 patients with primary atypical pneumonia

Pneumococcus		Beta hemolytic streptococcus			<i>H. influenzae</i>		Streptococcus MG
Type	Number of patients*	Group	Type	Number of patients*	Type	Number of patients	Number of patients
6	7	A	14, 15, 17	1	Could not be identified	34	45
3	6		19, C-94				
29	5		?	2			
9, 11, 16, 18, 20, 23	3						
4, 7, 8, 19, 28, 32, 37	2	B, C, H					
5, 10, 12, 17, 24, 25, 33, 34, 35, 36	1			1			
Total	26			10		34	45
Percentage of total patients	53.7			9.4		32.0	42.0

* Number of patients from whom each designated type was isolated.

of group A streptococci were represented. Strains of streptococci belonging to groups B, C, and H were each isolated from individual patients.

Strains of *H. influenzae* were present in cultures from 34 of the 106 patients but in no instance could the organisms isolated be identified by capsular swelling in immune rabbit sera against any of the established types.

B. friedländeri, type A, was isolated from the nasal passages of 1 patient, and *N. catarrhalis* in considerable numbers from 6 patients.

Staphylococci were frequently noted in cultures of the respiratory passages. They showed varying pigmentation and capacity to produce hemolysis on blood agar but only rarely did they appear to be the predominant organism in cultures from the patients in this study.

Colonies of organisms having the characteristics of alpha or gamma streptococci were frequently seen on blood agar plates inoculated with sputum or material from the nasopharynx. Appreciable numbers of organisms in these general categories were seen on blood agar cultures from 33 of the 106 patients. None of the many strains of alpha and gamma streptococci isolated by this method and tested by appropriate serological techniques could be identified as streptococcus MG.

Cultures of the blood from all patients showed no bacterial growth. Cultures of each available specimen of pleural and cerebrospinal fluid remained sterile.

In no instance was evidence obtained which indicated that any one of the bacterial species isolated from these patients by routine methods was causally related to the disease.

A non-hemolytic streptococcus, designated as streptococcus MG, was isolated from sputum or nasopharyngeal cultures of 45 of the 106 patients. In some instances, this streptococcus was cultured directly from the throat or from fresh sputum. It was also cultured, however, from source materials which had been stored at -70°C . for periods varying from a few weeks to more than 1 year. The special techniques for isolating streptococcus MG and the results of studies on this microorganism in primary atypical pneumonia are described in the accompanying paper (22).

VIRUS STUDIES

A continuous effort was made throughout this study to recover from specimens obtained from the patients in this series, as well as from specimens received from similar patients elsewhere, infectious agents other than bacteria which might be etiologically related to primary atypical pneumonia. A large number of specimens obtained from patients with this illness were inoculated in various animal species by several routes and, in many instances, serial passages were carried out. The specimens inoculated included sputa, nasopharyngeal washings, plasma, pleural, and cerebrospinal fluids, and suspensions of consolidated human lung from 9 fatal cases.

The animals used included mice, cotton rats, hooded rats, white rats, and hamsters which were inoculated by the intranasal, intracerebral, intraperitoneal, or subcutaneous route; guinea-pigs which were inoculated intraperitoneally and subcutaneously; rabbits which were inoculated intravenously, intraperitoneally, and subcutaneously; as well as monkeys, cotton rats, and hamsters, which were inoculated intratracheally and intranasally. Chick embryos at various stages of maturation were also inoculated by several techniques, including inoculation into the allantoic, amniotic, or yolk sacs, as well as directly upon the chorioallantoic membrane. Chick-embryo passage materials were inoculated in each of the animal species mentioned above as well as in chimpanzees.

With sputa obtained from certain patients, pulmonary consolidation was repeatedly induced following primary intranasal inoculation in cotton rats. Chick-embryo passage materials were also found to be capable of inducing pulmonary consolidation in either cotton rats or hamsters. Similar consolidations, however, were found to occur in both species following the intranasal inoculation of various control materials. The pulmonary lesions which were noted following inoculation with potentially infective specimens could not be distinguished from those which followed inoculation with control materials, and none of the lesions could be reproduced regularly on serial passage in either species. No evidence was obtained to indicate the presence, in any of the human source materials studied, of a non-bacterial infectious agent capable of producing signs of infection which could be reproduced in series.

Infectious agents, apparently harbored by animals used in these experiments, were encountered repeatedly and seriously complicated attempts to isolate an infectious agent from the human materials. Lymphocytic choriomeningitis virus, the pneumonia virus of mice (25), hereinafter referred to as P.V.M., as well as another latent virus of mice belonging to the psittacosis group of viruses (26), were recovered and identified. It was possible to show in each instance that these agents were derived from the mice used and not from the human materials.

Early in the course of this study, it was ob-

served (27) that cotton rats and rabbits inoculated with certain specimens from patients or with passage materials derived from them developed antibodies capable of neutralizing a heterologous virus, P.V.M., whereas animals inoculated with control materials did not. This evidence was at that time thought to indicate the presence, in these specimens from patients with primary atypical pneumonia, of a virus which possessed minor antigenic components in common with P.V.M.

Subsequent studies have necessitated reinterpretation of the original data. It has been found that in cotton rats and rabbits, as well as in hamsters, the formation of neutralizing antibodies against P.V.M. sometimes could be stimulated by the inoculation of non-infectious materials. Thus it appears that each of these animal species at times and under circumstances not as yet predictable may harbor latent agents which in response to non-specific stimulation induce the formation of specific antibodies against P.V.M. Under these circumstances it is apparent that an antibody response against P.V.M. in any of these species cannot be attributed to specific stimulation by a virus in material of human origin.

SEROLOGICAL STUDIES

Sera obtained from patients with primary atypical pneumonia during the acute and convalescent phases of the illness were tested for the presence of antibodies against a number of viruses known or thought to be capable of inducing acute respiratory disease in human beings. Tests of this nature were carried out upon sera, not only from the 106 patients admitted to the Rockefeller Hospital and described in this study, but also from a number of patients with primary atypical pneumonia treated elsewhere.

The agents used in these tests were influenza A virus, influenza B virus, swine influenza virus, psittacosis virus, and lymphocytic choriomeningitis virus. The results of these tests are shown in Table VI.

With but one exception, no significant increase in specific antibodies against any of these viruses was demonstrable in the serum obtained during convalescence as compared with the serum ob-

TABLE VI

Results of serological tests against certain pneumotropic viruses in patients with primary atypical pneumonia

Virus	Method	Number of patients tested			Results of tests	
		R.I.H.* patients	Outside† patients	Total patients	Significant increase in antibodies, No. of patients	No increase in antibodies, No. of patients
Influenza A	R.B.C.‡ agglutination	47	49	96	1**	95
Influenza B	R.B.C. agglutination	42	47	89	0	89
Swine influenza	R.B.C. agglutination	8	0	8	0	8
Psittacosis	Complement fixation	25	37	62	0	62
Lymphocytic choriomeningitis	Complement fixation	16	12	28	0	28

* R.I.H. patients = Patients studied in the Rockefeller Institute Hospital.

† Outside patients = Patients studied elsewhere.

‡ R.B.C. agglutination = agglutination of chick erythrocytes (Hirst technique).

** R.I.H. patient studied during the 1943-44 influenza A epidemic.

tained from the same patient during the acute phase of illness. A definite antibody response against influenza A virus occurred in the convalescent sera of one patient who had primary atypical pneumonia at the Rockefeller Institute Hospital during the outbreak of influenza A in the winter of 1943-44. The evidence obtained from serological studies strongly suggests that none of the viruses tested was causally related to the illness in the group of patients included in this investigation.

Tests for the presence of antibodies against *Rickettsia diaporica* were not carried out with sera from these patients. However, various specimens obtained from most of the patients were inoculated into susceptible animals and in no instance were rickettsiae recovered. The attempts of others (28) to isolate rickettsiae were unsuccessful also and antibodies against the rickettsia of Q fever were not demonstrable in the sera of any of their patients with primary atypical pneumonia.

In the course of testing sera from the patients in this study for complement-fixing antibodies, a peculiar and unexpected phenomenon was encountered (29). The convalescent sera from 14 of 35 patients tested showed the capacity, not present in acute-phase sera, to fix complement with a variety of apparently unrelated antigens prepared from normal and infected organs of several different species. Similar reactions were not observed in comparable sera from 23 patients with other acute infectious diseases. This phenomenon obviously complicates the interpretation of complement-fixa-

tion tests in primary atypical pneumonia. In the light of these observations, it seems necessary to re-evaluate the significance of serological studies in this disease which were dependent upon results obtained with the complement-fixation technique when relatively crude tissue antigens were employed.

Sera from 18 of the patients with primary atypical pneumonia were also tested for the presence of the so-called "C-reactive protein" (30). It will be recalled that notably in pneumococcal pneumonia, but also in other acute infectious diseases, there is present in the serum during the acute phase of illness an abnormal protein which has the peculiar property of reacting with the C or somatic polysaccharide of the pneumococcus. It was found that this protein was present in the acute-phase serum of all but one of the patients with primary atypical pneumonia whose sera were tested, and that it disappeared from their sera during convalescence, as it does in other acute infectious diseases.

Sera obtained from 64 of these patients throughout the course of primary atypical pneumonia were tested for the presence of cold hemagglutinins against human Group O erythrocytes (31, 32). It was found that sera of 12, or 18.5 per cent, showed this property.

Reports from other laboratories (31 to 36) have recorded a variable although generally higher incidence of cold hemagglutinins in sera of patients with this disease. The lower percentage of positive reactions and the relatively low titers with sera of patients in this group may be attributable to the method used in collecting some

of the sera, to prolonged storage of certain of the sera before testing, and possibly to the fact that many of the patients in this series were only mildly ill.

Prior to the observation of cold hemagglutinins in this disease, some of the specimens of blood obtained from patients in this series were allowed to clot at 4° C. before the serum was separated. Subsequently, it was found that hemagglutinins present in high titer could be completely absorbed from a specimen of serum by contact with a suspension of human erythrocytes at a temperature of 4° C. It seemed advisable therefore to allow blood specimens to clot at room temperature and to separate the serum prior to refrigeration.

It has been noted previously that in sera obtained from patients with primary atypical pneumonia and stored for long periods of time, the capacity for cold hemagglutination appeared to be less than in sera recently obtained from similar patients (31, 33). One worker (35) retested 20 specimens of serum after storage at 4° C. for periods ranging from 2 to 5 months and found that, in all but 1, the titer of cold agglutinins had diminished. Seven specimens of serum from patients in the present series were likewise tested on 2 occasions with similar results. At the time of the initial tests, the sera had been stored for periods varying from 2 weeks to 6 months but all showed cold agglutination, in dilutions ranging from 1:40 to 1:160. When tested again 10 or 11 months later, 2 sera agglutinated erythrocytes with an undiminished titer of 1-80, 2 agglutinated only in a dilution of 1-10, and 3 had lost completely the property of cold agglutination.

Tests for cold hemagglutination were carried out upon sera of patients in the present series using 2.0 per cent suspensions of erythrocytes as originally recommended. Ten of the 12 patients in this series whose sera contained cold hemagglutinins were ill for periods longer than the average duration of illness for the total group. Other observers have also noted a positive correlation between either the incidence or the titer of cold hemagglutinins and the severity of illness in patients with primary atypical pneumonia (31, 36).

Acute and convalescent sera from each of the

106 patients were tested for the presence of agglutinins against the non-hemolytic streptococcus designated as streptococcus MG (22). It was found that 68, or 64 per cent, of the 106 patients developed in their sera during convalescence agglutinins against this microorganism.

The incidence of positive reactions in agglutination tests with streptococcus MG and serial specimens of serum from these 68 patients (MG positive group) is shown in Figure 1. Although

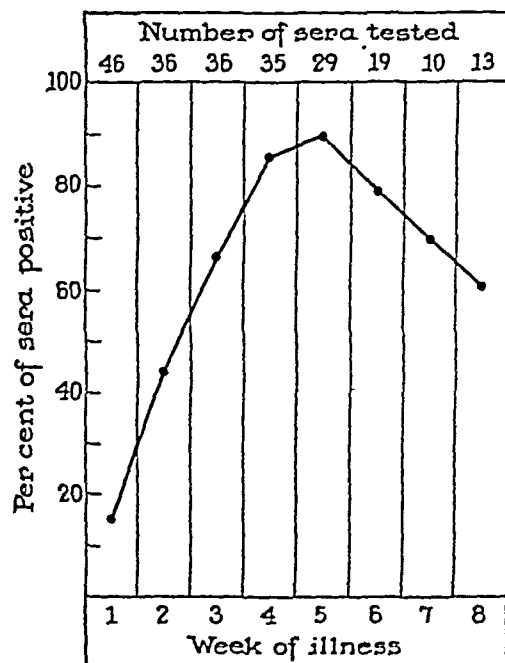


FIG. 1. TIME OF OCCURRENCE OF POSITIVE REACTIONS IN AGGLUTINATION TESTS WITH STREPTOCOCCUS MG AND SERA FROM 68 SELECTED PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA (MG-POSITIVE GROUP)

Each patient in this group developed in his serum during convalescence agglutinins against streptococcus MG. Only one specimen of serum per patient per week was included in this analysis.

positive reactions were noted in 15, 44, and 67 per cent of the sera obtained from these patients during the first, second, and third weeks of illness, respectively, they were found in 86 and 90 per cent of the sera obtained during the fourth and fifth weeks of illness. In 58 (85 per cent) patients, agglutinins were first detected in specimens of serum obtained on the day of defervescence or later in convalescence. In the sera of 10 patients (15 per cent), agglutinins were

noted from 2 to 19 days before defervescence, but the maximum titer of the sera of all but 3 of these patients was not attained until after the temperature had returned to normal. It may be said, therefore, that agglutinins against streptococcus MG are most frequently detectable in sera of patients with primary atypical pneumonia 4 to 5 weeks after the onset of illness and, in most instances, make their first appearance following defervescence.

In view of the frequency with which a serological response against streptococcus MG was observed in these patients, it seemed of interest to determine in what other respects the 68 patients who developed antibodies against this microorganism (MG positive group) could be distinguished from the 38 patients in whose sera such antibodies were not demonstrable (MG-negative group).

On the basis of clinical impressions, the MG-positive group seemed to include patients who were more ill than most patients in the MG-negative group but no satisfactory quantitative criteria except those relative to the duration of the illness could be found for estimating the relative severity of illness in individual patients of the 2 groups. From the standpoint of severity, the symptoms, physical signs, and clinical-laboratory findings of many individual patients in the 2 groups were indistinguishable. The average number of lobes involved was by physical examination 1.64 and 1.39 and by x-ray 2.01 and 1.55 in the MG-positive and MG-negative groups, respectively. On the basis of x-ray evidence, involvement of more than one lobe occurred in 56 per cent of the MG-positive group and in 47 per cent of the MG-negative group. The average and range of the total leukocyte counts, the degree and frequency of E.S.R. acceleration, the distribution of bacteria other than streptococcus MG, and the negative results of serological tests against certain pneumotropic viruses were essentially the same in both groups.

Nevertheless, it was possible to demonstrate certain quantitative differences between patients in the 2 groups taken collectively if data relating to the duration of illness were compared. It was found that the duration of fever, of abnormal physical signs, and of roentgenological evidence of pneumonia, as well as the duration of hospital-

ization, were considerably longer in the MG-positive group than in the MG-negative group.

Comparable data for the 2 groups are illustrated graphically in Figure 2. Fever persisted

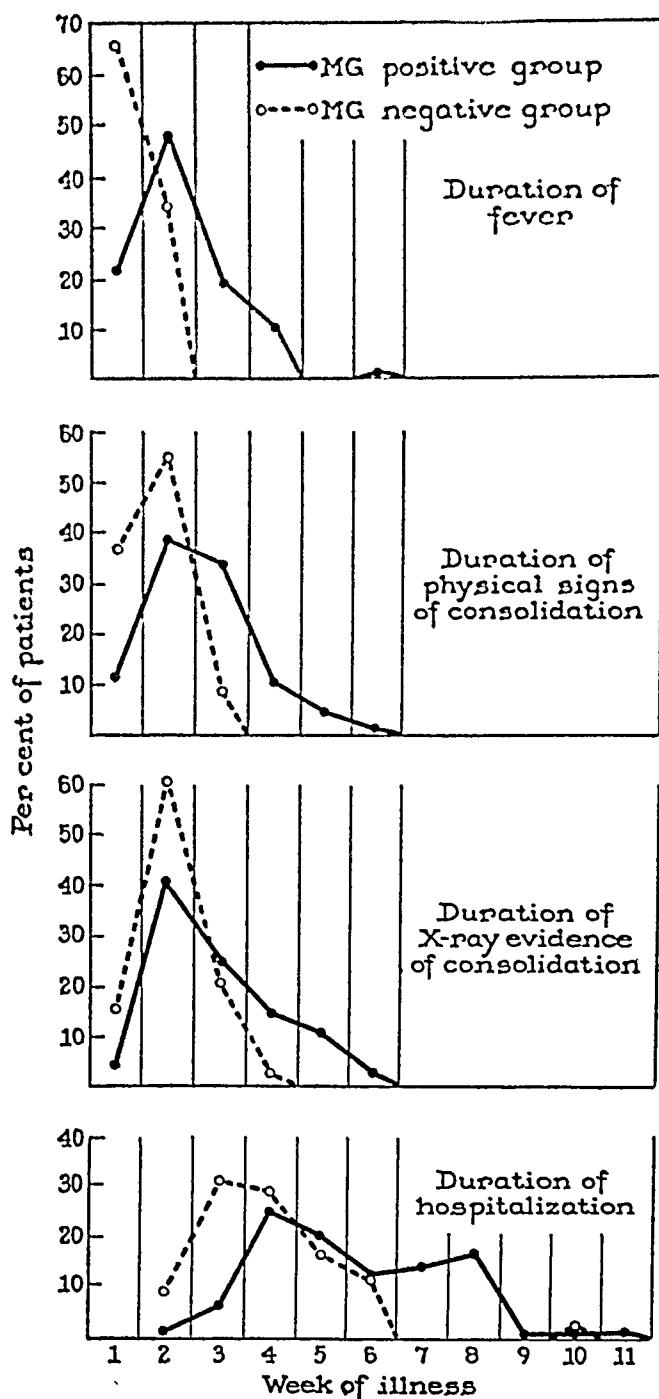


FIG. 2. COMPARISON OF QUANTITATIVE DATA RELATIVE TO THE DURATION OF ILLNESS IN THE MG-POSITIVE AND MG-NEGATIVE GROUPS OF PATIENTS WITH PRIMARY ATYPICAL PNEUMONIA

MG-positive group comprises those patients who developed in their serum agglutinins against streptococcus MG. MG-negative group is composed of those patients in whose serum such agglutinins were not demonstrable.

for more than 2 weeks in 21 patients (30 per cent) of the MG-positive group and in none of the patients in the MG-negative group. Physical signs of pneumonia were present for more than 2 weeks in 34 patients (50 per cent) of the MG-positive group and in only 3 patients (7 per cent) of the MG-negative group. Roentgenological evidence of pneumonia was detectable for more than 3 weeks in 20 patients (29 per cent) of the MG-positive group and in only 1 patient of the MG-negative group. Similarly, 23 patients (34 per cent) of the MG-positive group but only 1 patient of the MG-negative group required hospitalization for more than 6 weeks.

Further analysis revealed that the average duration of illness was longer, not only in the MG-positive group as compared with the MG-negative group, but also within the MG-positive group in direct proportion to the maximum titer of agglutinins against streptococcus MG. This is shown in Table VII. The duration in days of

physical signs of pneumonia for 8.9 days, evidence of pneumonia by x-ray for 11.4 days, and hospitalization for 26.2 days. In the groups of patients with maximum agglutinin titers in their sera of 1-10, 1-20, and 1-40 or more, respectively, the average duration of illness was progressively longer in each of the 4 categories mentioned above. Thus, the 20 patients in whose sera the maximum titer of agglutinins (against streptococcus MG) was 1-40 or more the average duration of fever was 17.3 days, signs of pneumonia were present for an average of 20.4 days, and evidence of pneumonia by x-ray for 23 days, while the average period of hospitalization was 46.3 days. It should be pointed out that within the groups of patients divided according to the titer of their sera against streptococcus MG, the duration of illness in individual patients varied over a wide range. Moreover, as might be expected, there was considerable overlapping in the range of the values among the several titer groups. Nevertheless, these observations showed in patients of this series a positive correlation between the duration of illness and the titer of agglutinins against streptococcus MG.

The MG-positive and MG-negative groups of patients also differed markedly with respect to the occurrence in their sera of cold hemagglutinins. The results of serological tests for both cold hemagglutination and agglutination of streptococcus MG are shown in Table VIII.

TABLE VII
Relation of duration of illness to the titer of agglutinins against streptococcus MG in the sera of patients with primary atypical pneumonia

Maximum titer of agglutinins against streptococcus MG	Number of patients	Duration of illness			Duration of hospitalization
		Fever	Physical signs of pneumonia	X-ray evidence of pneumonia	
		Average number of days	Average number of days	Average number of days	Average number of days
0	38	6.6	8.9	11.4	26.2
1-10	28	9.4	11.6	14.1	30.5
1-20	20	10.8	14.7	15.8	35.4
1-40 and over	20	17.3	20.4	23.0	46.3
Total group	106	10.1	12.8	14.2	33.0

fever, of physical signs of pneumonia, of x-ray evidence of pneumonia, and the period of hospitalization were arbitrarily estimated for each patient from the onset of illness. The average duration in each of these 4 categories was then calculated in groups of patients arranged according to the maximum titer of agglutinins against streptococcus MG observed in their sera throughout the course of illness.

As shown in Table VII, 38 patients with no demonstrable agglutinins against streptococcus MG had, on the average, fever for 6.6 days,

TABLE VIII
Results of tests for cold hemagglutination and for agglutination of streptococcus MG with sera of patients with primary atypical pneumonia

Cold hemagglutination	Agglutination of streptococcus MG		Total number of patients
	Positive—Number of patients	Negative—Number of patients	
Positive	13	3	16
Negative	19	36	55
Total	32	39	71

Sera from a total of 71 patients, including 64 patients in the present series and 7 patients studied elsewhere,¹ were tested for both types of agglu-

¹ Dr. Winfield T. Lothrop of the Johns Hopkins Hospital, Baltimore, Md., has graciously loaned the serum from these 7 patients.

tinins. Convalescent sera from 32 of these 71 patients agglutinated streptococcus MG. Of these 32 patients, the sera of 13 also agglutinated human Group O erythrocytes in the cold. The sera of 36 patients possessed neither agglutinins against streptococcus MG nor cold hemagglutinins. The sera of 3 patients possessed cold hemagglutinins but not agglutinins against streptococcus MG. Thus it is evident that cold hemagglutinins occurred predominantly but not exclusively in the sera of patients who developed agglutinins against streptococcus MG.

In the 12 patients of the present series whose sera contained cold hemagglutinins, the average duration of fever was 16.4 days, pneumonia was detected by physical signs for an average of 20.2 days, and by x-rays for an average of 20.7 days; the average duration of hospitalization was 44.8 days. It is evident that the average duration of illness in the group of patients with cold hemagglutinins corresponded closely to that observed in the group of patients in whose sera the titer of agglutinins against streptococcus MG was 1-40 or more.

It was pointed out previously that the convalescent sera from 14 of the patients in the present series were capable of fixing complement with a variety of apparently unrelated tissue antigens. All but 1 of the patients in whose sera this property was demonstrated were in the MG-positive group. The sera of five also contained cold hemagglutinins. The average duration of fever in these 14 patients was 13.2 days. Pneumonia was detectable by physical signs for an average of 18.5 days and by x-ray for 17.3 days. The average period of hospitalization was 37.7 days. Thus, the average duration of illness in these patients was considerably longer than the average duration of illness for all the patients and was similar to that observed, not only in patients who had higher titers of agglutinins against streptococcus MG, but also in the group of patients with cold hemagglutinins.

These findings indicate that agglutinins against streptococcus MG, cold hemagglutinins, and the property of non-specific complement-fixation may coexist in specimens of serum from individual patients with primary atypical pneumonia. Moreover they strongly suggest that there is a positive correlation between the inci-

dence of each of these serological properties and the duration of the illness. In the accompanying paper (22), evidence is presented which shows that, despite these similarities, the 3 phenomena are distinct and can be clearly differentiated.

DISCUSSION

During the past 10 years, the illness which has been designated by the term "primary atypical pneumonia" has become a familiar clinical entity which can be recognized on the basis of certain diagnostic criteria. It is possible that this disease may have existed at the time of the last war and even earlier. However, as the validity of diagnosis is dependent upon the careful exclusion of other diseases and to a considerable degree upon the results of roentgenological examinations, there is a limit beyond which retroactive diagnosis becomes merely speculation.

The occurrence of primary atypical pneumonia in certain members of a community in which undifferentiated forms of acute respiratory infection are prevalent has been frequently observed and suggests that this form of pneumonia may be but one manifestation of a widespread respiratory disease. This view is supported by epidemiological observations (37).

In making the diagnosis of primary atypical pneumonia, certain clinical features are particularly helpful. The gradual onset of malaise, cough, headache, and fever, the lack of respiratory distress, the relative bradycardia, the normal leukocyte count, and the paucity of pulmonary signs on physical examination in the presence of x-ray evidence of pneumonia are sufficiently frequent and characteristic findings of this disease syndrome to warrant a presumptive diagnosis.

X-ray evidence of pneumonia may not necessarily be present at the initial examination but usually appears early in the course of illness and clears completely within a few weeks. The x-ray shadow frequently is diffuse and radiates from the hilum but may assume a variety of patterns and, at times, is indistinguishable from that observed in other diseases, particularly other varieties of pneumonia, bronchiectasis, and tuberculosis. The duration of illness should be considered in interpreting x-ray films of the chest in patients with this syndrome and the

rate of clearing is of particular value in excluding a diagnosis of chronic pulmonary disease.

By appropriate techniques, pneumococci can frequently be recovered from patients with the characteristic features of primary atypical pneumonia. It is unusual however to find them in this disease by direct examination of sputum, employing the quellung technique. Moreover, the pneumococcal types isolated are rarely those most frequently associated with lobar pneumonia. It should be pointed out, however, that during the administration of sulfonamide drugs, a considerable reduction in the number of susceptible microorganisms in the nasopharynx and sputum may take place. As a consequence, the presence of certain pathogenic microorganisms, particularly pneumococci or beta hemolytic streptococci, may not be detected. It is therefore important in distinguishing this syndrome from other forms of pneumonia, that the bacteriological examination be carried out whenever possible before sulfonamide therapy is instituted.

The sulfonamide drugs have not been found to be effective in primary atypical pneumonia and are not recommended for treatment. In certain instances, however, it may be impossible to determine whether or not pneumococci or hemolytic streptococci isolated from a patient are implicated in the pathogenesis of the illness. Under such circumstances, an adequate trial of sulfonamide therapy is justified. Prompt defervescence following the administration of a sulfonamide drug may be an indication of effective therapy but may occur also as a coincidence. Consequently, an apparent response to sulfonamide therapy does not necessarily exclude a diagnosis of primary atypical pneumonia.

Early reports on the occurrence of cold hemagglutinins in the sera of patients with primary atypical pneumonia (31 to 33) indicated that this phenomenon might provide a technique useful in diagnosis. It is now apparent, however, that many patients with primary atypical pneumonia do not develop in their sera the capacity to agglutinate erythrocytes in the cold. Furthermore, it has been found that this serological property occurs not only in patients with primary atypical pneumonia but also in an appreciable number of patients with other conditions. Al-

though the significance of the phenomenon has yet to be ascertained, its value as a practical laboratory aid to diagnosis has been disappointing.

Another phenomenon which has been observed with the convalescent sera of certain patients with primary atypical pneumonia is the capacity to fix complement with unrelated tissue antigens (29, 38). This property also does not occur with sufficient frequency to be reliable as a diagnostic technique. Nevertheless, its recognition has served to demonstrate further the frequency with which non-specific reactions occur in the sera of patients with primary atypical pneumonia. It also has made necessary a more critical evaluation of serological data based upon the results of complement-fixation tests in studies of this disease.

It has been pointed out that the clinical picture of primary atypical pneumonia may represent instances of infection by different etiological agents. In certain patients, *Rickettsia diaporica* (11) and the psittacosis group of viruses (12, 13) have been shown to be responsible. These agents, however, have been isolated from or otherwise identified with relatively few cases. In the present series of patients, it was found that neither of these agents had caused the disease and ample evidence is now available to indicate that they were not responsible for the great majority of cases which have been studied during recent years.

The recovery of several infectious agents from patients with primary atypical pneumonia has been reported. A virus obtained by one group of workers (39) appeared to be infectious for mice, guinea pigs, and ferrets, but was lost after a few passages. Others (14) described the recovery of a virus which was infectious for the mongoose, and presented evidence indicating that the agent could be neutralized by the sera of patients convalescent from primary atypical pneumonia. Unfortunately, this species was not available for an attempt to repeat these observations with materials from patients in the present series.

Two viruses recovered from cats have been described in relation to primary atypical pneumonia in man. Epidemiological evidence has been presented (16) to indicate that a few cases

of the human disease in one family might have been related to a respiratory infection of cats which was caused by a virus apparently infectious for this species but not for mice. This agent is different from the other feline virus, recently isolated (40), which has been shown to belong to the psittacosis-lymphogranuloma group of viruses (41).

Certain investigators (15) observed that pulmonary consolidation developed in cotton rats following primary intranasal inoculation of sputum from certain patients with primary atypical pneumonia and presented evidence for the existence of a transmissible agent. Recently (18), these workers have reported the recovery of a filterable agent from certain patients with atypical pneumonia. They also reported that this agent was transmissible in chick embryos and was capable of inducing pulmonary lesions after intranasal inoculation in either cotton rats or hamsters. Moreover, evidence was presented to show that the agent could be neutralized by the convalescent serum but not the acute-phase serum from patients with primary atypical pneumonia. They also reported that pulmonary lesions were induced in both cotton rats and hamsters by the intranasal inoculation of non-infectious materials and that these lesions were similar to those induced by the agent. Furthermore, they found that latent agents present in both species were encountered frequently when serial passages were carried out.

As has been pointed out, the agent previously described in a report from this laboratory (27) was found on further study to be indistinguishable from latent agents antigenically related to the pneumonia virus of mice (25). In the course of the present study, evidence has been obtained which indicates that such latent agents are present in cotton rats, hamsters, and rabbits.

A filterable virus recovered from patients with primary atypical pneumonia (17) was found to be transmissible on intranasal inoculation in young guinea-pigs and cotton rats. The results of neutralization tests with human sera were inconclusive. It was found (18) that guinea-pigs frequently developed lung lesions after intranasal inoculation of broth and one agent was recovered, by intranasal passage in guinea-

pigs, which produced pulmonary consolidation in cotton rats and hamsters and resembled an agent obtained from normal hamsters.

The significance of any of these viruses in the pathogenesis of primary atypical pneumonia as observed in the majority of patients remains to be determined.

As reported previously (19), a non-hemolytic streptococcus, isolated originally from the lungs of patients who had died of primary atypical pneumonia, was found to react with the sera of many patients convalescent from this disease. The characteristics of this microorganism, which has been designated streptococcus MG, have been investigated intensively. The results of these studies are presented in separate communications (20, 21). In the accompanying paper (22), studies concerning the relation of streptococcus MG to primary atypical pneumonia are presented in detail and the possible rôle of this microorganism in the pathogenesis of the disease is discussed.

SUMMARY

A comprehensive study of 106 patients with primary atypical pneumonia is presented. The study includes observations on clinical characteristics, roentgenological, electrocardiographic, and clinical-laboratory findings, as well as the results of biochemical, bacteriological, viral, and serological investigations. The observations made upon patients in this series are correlated with the findings reported in other studies on this disease.

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STUDIES ON PRIMARY ATYPICAL PNEUMONIA.

II. OBSERVATIONS CONCERNING THE RELATIONSHIP OF A NON-HEMOLYTIC STREPTOCOCCUS TO THE DISEASE^{1,2}

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In a preliminary communication from this laboratory (1), the isolation of a non-hemolytic streptococcus from the lungs of 2 fatal cases of primary atypical pneumonia was reported. It was shown that convalescent sera of numerous patients with primary atypical pneumonia were capable of agglutinating this microorganism and some sera yielded precipitates with appropriate extracts of it. It was also shown that, except in rare instances, similar reactions were not demonstrable with acute-phase sera from the same patients, sera from patients with other acute infectious diseases, or sera from normal individuals.

Since the publication of these preliminary observations, additional strains of this non-hemolytic streptococcus have been isolated from the lungs and sputa of other patients with primary atypical pneumonia. A comprehensive study of the biological and immunological characteristics of this microorganism has been undertaken, and the results are described in detail in separate communications (2, 3). The serological reactions obtained with this streptococcus have been investigated, with additional specimens of serum from patients with primary atypical pneumonia, patients with other diseases, and normal persons, by means of a number of different immunological procedures. Of the patients with primary atypical pneumonia, 106 were studied in the Hospital

of The Rockefeller Institute, and an analysis of the clinical, roentgenological, and laboratory findings in these patients is presented in the accompanying paper (4).

It is the purpose of the present paper to summarize the information which has been obtained concerning certain properties of this streptococcus, the methods by which it was isolated from lung tissue, sputum, and throat swabs, and the serological reactions which were demonstrable with the convalescent serum of patients with primary atypical pneumonia. Evidence has been obtained which indicates that the strains of this microorganism which have been studied constitute a homogeneous group and belong to a single serological type, and may readily be differentiated from other varieties of non-hemolytic streptococci. It will be shown that the positive serological reactions obtained with this streptococcus and convalescent serum from patients with primary atypical pneumonia were due to specific antibodies which developed during the course of the illness. Moreover, evidence will be presented which indicates that these antibodies were independent of the properties of non-specific complement fixation (5) and cold hemagglutination (6, 7), which were also exhibited by certain of the convalescent sera studied.

For convenience, this streptococcus will be referred to in this report as "streptococcus MG." The problem of classification of streptococcus MG is discussed in a separate paper (2).

MATERIALS AND METHODS

Specimens

Specimens of sputum from patients with primary atypical pneumonia were frozen rapidly at -70°C . Sputa from patients with other acute infections of the respiratory tract were treated in an identical manner.

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Lung tissues from fatal cases of primary atypical pneumonia and from patients who died of other conditions were also stored at -70°C .⁵ These various specimens were stored for periods ranging from a few days to 2 years before they were cultured. In addition to the frozen materials, fresh specimens of sputum and throat swabs from patients and normal persons were also cultured.

Culture media

For the isolation of streptococcus MG from specimens of lung tissue, Brewer's thioglycollate broth was employed (8). This medium was unsatisfactory for the culture of sputum specimens or throat swabs because of the rapid overgrowth of streptococcus MG by other microorganisms. For the isolation of streptococcus MG from such specimens, a semiselective medium was employed which permitted the growth of this microorganism and other varieties of non-hemolytic streptococci but inhibited the growth of most other bacterial species. This medium, which was similar in some respects to that recommended (9) for the selective cultivation of *Streptococcus salivarius*, was composed of the following constituents:

Proteose peptone	Difco	5 grams
Yeast extract	Bacto	5 grams
Beef extract	Bacto	3 grams
Glucose	C. P.	10 grams
Gentian violet		2 mgm.
Sodium azide		200 mgm.
Sulfapyridine		500 mgm.
Distilled water		1000 ml.

The pH of the completed medium was adjusted to 7.2 with 1N NaOH and the medium was then sterilized in the autoclave at 15 pounds pressure for 15 minutes.

For the maintenance and continued subculture of streptococcus MG in the laboratory, Todd-Hewitt broth was employed. When cultures were stored at 4°C . for periods of longer than 1 week, defibrinated rabbit blood was added to the broth in a concentration of 2 per cent. Blood broth cultures of streptococcus MG remained viable for as long as 2 months at 4°C .

Nutrient agar containing 5 per cent sucrose was employed for the differentiation of streptococcus MG from *Str. salivarius*. The latter organism has been shown to produce very large, mucoid colonies on agar containing 5 per cent sucrose (10). In contrast, streptococcus MG grew on this medium in the form of small fluorescent colonies.

Rabbit antisera

Antiserum was prepared by the intravenous immunization of rabbits with saline suspensions of heat-killed streptococcus MG. The details of the procedure used for

immunization are described elsewhere (3). The immune sera thus prepared usually possessed agglutination titers of 1:2500 or higher, and also caused definite capsular swelling of streptococcus MG.

Procedures for isolation and identification

A loopful of sputum was inoculated into approximately 4 ml. of the semiselective medium described above, and incubated at 37°C . for 24 to 48 hours. Throat swabs were placed directly in 4 ml. of this medium and incubated in a similar manner. Lung tissue suspensions were cultured in Brewer's thioglycollate medium.

It should be noted that streptococcus MG grew very poorly, or not at all, when suspensions of lung tissue containing it were streaked on the surface of blood agar plates, either under aerobic or anaerobic conditions. The reason for this is not clear, since broth cultures of streptococcus MG grew readily when inoculated on blood agar, and formed small, grey, coniform colonies. The failure to obtain growth with direct cultures of lung tissue on blood agar was apparently not due merely to the presence of small numbers of streptococci in the lung tissue suspension, since, as will be shown below, these suspensions even when diluted to 10^{-3} yielded growth in thioglycollate medium.

When visible growth in broth had occurred, a loopful of the culture was mixed with a loopful of rabbit anti-streptococcus MG serum together with a loopful of 1 per cent methylene blue, and was then examined for capsular swelling. A positive quellung reaction was taken to indicate the presence of either streptococcus MG or *Str. salivarius*, type I. Evidence is presented elsewhere (3) which shows that the latter microorganism possesses certain antigenic components also present in streptococcus MG.

In order to differentiate streptococcus MG from *Str. salivarius*, type I, the cultures were then inoculated on the surface of 5 per cent sucrose agar plates. Single samples of the small, fluorescent colonies formed on this medium by streptococcus MG were inoculated in beef-infusion broth. Following growth in broth, the cultures were again tested for quellung in rabbit anti-streptococcus MG serum.

ISOLATION OF STREPTOCOCCUS MG FROM LUNG TISSUE

Lung tissues from 8 fatal cases of primary atypical pneumonia, and from 6 patients who died of other causes were cultured by the methods described above. The results are shown in Table I. Streptococcus MG was isolated from 6 of the 8 specimens of lung tissue from patients with primary atypical pneumonia. In contrast, this microorganism was not isolated from any of the 6 lung specimens from patients with other diseases, including 1 case of psittacosis, 1 of traumatic death, and 4 of pneumonia due

⁵ The authors are grateful to Dr. A. A. Applebaum, Toledo, Ohio, Major J. H. Dingle, M.C., A.U.S., Fort Bragg, N. C., Dr. W. S. Tillett, New York, N. Y., Commander J. A. de Veer, M.C., U.S.N.R., New York, N. Y., and Dr. W. C. Von Glahn, New York, N. Y., who generously supplied specimens of lung tissue.

TABLE I

Isolation of streptococcus MG from the respiratory tract of human beings

Specimen	Diagnosis	Number patients	Positive cultures	
			Number	Per cent
Lung	Primary atypical pneumonia	8	6	75
	Other diseases*	6	0	0
Sputum or throat swab	Primary atypical pneumonia	97	53	55
	Acute respiratory infection without pneumonia	39	13	33
	Pneumococcal pneumonia	19	4	21
	Other bacterial pneumonias	15	3	20
	Influenza A	9	0	0
	Normal persons	57	7	12.5

* 1 psittacosis, 1 trauma, 4 bacterial pneumonia.

to other varieties of bacteria. The 6 patients with primary atypical pneumonia whose lung tissues yielded positive cultures died between the 8th and 22nd day after the onset of the disease, while the 2 negative specimens were from patients who died on the 25th and 34th days, respectively, after onset.

Str. salivarius was not encountered in any of the cultures of lung tissue from patients with primary atypical pneumonia. Four of the specimens contained small numbers of other bacteria which could be eliminated from the cultures by preparing higher dilutions of the suspensions of lung tissue before culturing in thioglycollate broth. It is noteworthy that pure cultures of streptococcus MG were obtained from 4 of the lung specimens when dilutions as high as 10^{-5} were cultured.

Occasional colonies of the strains of non-hemolytic streptococci isolated from lung tissue were encountered which did not exhibit quellung in the presence of rabbit anti-streptococcus MG serum. When grown in broth, subcultures from these particular colonies produced a flocculent type of growth, rather than the diffuse type of growth characteristic of streptococcus MG. Further comments on these strains will be found

below under the heading: *Dissociation of streptococcus MG*.

ISOLATION OF STREPTOCOCCUS MG FROM SPUTUM

Specimens of sputum or throat swabs from patients with primary atypical pneumonia, patients with other infectious diseases, and normal persons were cultured in the semiselective medium by the methods described above. The results are included in Table I. Streptococcus MG was isolated from 53 of a total of 97 specimens from patients with primary atypical pneumonia. This microorganism was also recovered from 13 of 39 specimens from patients with acute upper respiratory infections without pneumonia, or so-called "catarrhal fever," from 4 of 19 specimens from patients with pneumococcal pneumonia, and from 3 of 15 specimens from patients with other types of bacterial pneumonia, but was not obtained from the sputum of 9 patients with influenza A. Streptococcus MG was isolated from the throat cultures of 7 of a total of 57 normal persons.

BIOLOGICAL CHARACTERISTICS OF STREPTOCOCCUS MG

A total of 59 strains of streptococcus MG have been subjected to a detailed investigation. Each of these strains was selected on the basis of the following criteria: (1) quellung in rabbit anti-streptococcus MG serum, and (2) the formation of small fluorescent colonies on 5 per cent sucrose agar. Thirty-three of these strains were also tested by the agglutination technique with acute-phase and convalescent serum from selected patients with primary atypical pneumonia. Each of these strains was agglutinated by convalescent serum but not by acute-phase serum. The characteristics of the agglutination reactions with these strains were identical to those noted with the first strains of streptococcus MG isolated (1).

None of the 59 strains produced greening or hemolysis of rabbit blood agar when incubated for 24 hours. However, longer periods of incubation resulted in the production of distinct greening by some strains. Several strains produced more definite greening when grown on horse or sheep blood agar.

A detailed account of the biological properties

of streptococcus MG is given in a separate paper (2). It was found that there was a striking homogeneity in the properties of different strains of streptococcus MG, and that these properties made it possible to differentiate this microorganism from other non-hemolytic streptococci. It was also found that streptococcus MG was not pathogenic for any of the common laboratory animals. It was highly resistant to bile, and to the bacteriostatic action of sulfapyridine, as tested by the method of MacLeod and Mirick (11). It was however, susceptible to the effect of penicillin.

Effect of storage at -70°C .

Most of the specimens of lung tissue and sputum from patients with primary atypical pneumonia had been stored at -70°C . for periods varying from a few days to two years. It was therefore of interest to determine the effect of storage under these conditions upon freshly isolated cultures of streptococcus MG. Young broth cultures of 19 strains of this bacterium were stored at -70°C . for 3 months. At the end of this time, subcultures from these tubes were made in beef-infusion broth and in gentian violet-sodium azide-sulfapyridine broth. It was found that 3 of the strains failed to grow in either medium, and that 7 others grew in beef-infusion broth but failed to grow in the semi-selective medium. With 5 of the broth subcultures, capsular swelling in the presence of rabbit antiserum could no longer be demonstrated. The adverse effect of storage of streptococcus MG under these conditions suggests that this microorganism may be more readily isolated from freshly obtained specimens of sputum or lung tissue than from specimens which have undergone prolonged storage at -70°C .

Soluble specific substance of streptococcus MG

When sterile filtrates of broth cultures of streptococcus MG were mixed with homologous immune rabbit serum, definite precipitation occurred. Precipitation did not occur when such filtrates were mixed with normal rabbit serum or with the serum of rabbits immunized with pneumococci or beta-hemolytic streptococci. This finding together with the positive quellung reactions obtained with homologous immune

rabbit serum suggested that streptococcus MG might possess a capsular polysaccharide analogous to those of type-specific pneumococci. Evidence has been obtained which indicates that streptococcus MG does possess a capsular polysaccharide and that the type-specific immunological reactions obtained with this microorganism are dependent upon the presence of this substance (3). The results of precipitation tests with the capsular polysaccharide and serum from patients with primary atypical pneumonia, intradermal tests with the substance in patients with this illness, and tests for the antigenicity of the polysaccharide in normal human beings will be given below.

Dissociation of streptococcus MG

By means of prolonged cultivation in broth containing homologous immune rabbit serum, dissociation of streptococcus MG was induced. The so-called R variants obtained by this procedure no longer were capable of synthesizing demonstrable capsular polysaccharide, and failed to give a positive quellung reaction with rabbit anti-streptococcus MG serum (3). The serum of rabbits immunized with R variants was capable in high dilution of agglutinating the R variants but either failed to agglutinate encapsulated strains of streptococcus MG or agglutinated them only at very low serum dilutions. In their biological and immunological properties, these R variants closely resembled certain single colony strains described above, which were obtained from the lung tissues of fatal cases of primary atypical pneumonia and which were negative in the quellung test. The available evidence suggests the possibility that these latter strains may represent similar non-encapsulated variants of streptococcus MG.

Antigenic relationship of streptococcus MG to Streptococcus salivarius, type I

The results obtained in studies on the biological properties of streptococcus MG (2), indicated clearly the marked differences between the characteristics of this microorganism and those of the *Str. salivarius* group. One striking and easily recognized differential property is their colonial morphology on nutrient agar containing 5 per cent sucrose. As was stated above, strep-

tococcus MG produced only small fluorescent colonies on this medium, whereas *Str. salivarius* produced very large mucoid colonies. In contrast with the significant differences in the biological properties of these microorganisms, evidence also has been obtained which indicates that streptococcus MG possesses certain antigenic components in common with *Str. salivarius*, type I (3). Various serological cross-tests revealed antigenic similarities between the capsular polysaccharides of these 2 different species of non-hemolytic streptococci. However, cross absorption tests with immune rabbit sera showed that although streptococcus MG and *Str. salivarius*, type I, were related antigenically they were not antigenically identical and could be distinguished from each other in immunological tests with appropriately absorbed antisera. It should be pointed out that streptococcus MG was not immunologically related to *Str. salivarius*, type II (3).

SEROLOGICAL REACTIONS WITH STREPTOCOCCUS MG

Agglutination tests with streptococcus MG were carried out with all available sera from 193 patients with primary atypical pneumonia. Of these patients, 106 were studied in the Hospital of The Rockefeller Institute, and the remaining 87 patients were studied in other hospitals.⁶ As controls, similar tests were performed with sera from 321 normal persons and 120 patients with other diseases, including psittacosis, pneumococcal pneumonia, acute respiratory infections without pneumonia ("catarrhal fever"), scarlet fever, influenza A, rheumatic fever, and other streptococcal infections. Agglutination tests were also carried out with sera from 9 normal human beings who were vaccinated intradermally with the capsular polysaccharide of streptococcus MG, and from 2 normal human beings who were vaccinated subcutaneously with a saline suspension of heat-killed streptococcus MG.

Selected specimens of serum were also tested by precipitation and complement fixation tech-

⁶ The authors are grateful to Dr. G. P. Berry, Rochester, N. Y., and Dr. W. T. Longcope, Baltimore, Md., who generously supplied specimens of sera from their patients with primary atypical pneumonia.

niques against the capsular polysaccharide of streptococcus MG, and by the quellung technique with the intact microorganism. Skin tests with this polysaccharide were performed in 22 patients with primary atypical pneumonia, 14 patients with other diseases, and 14 normal persons.

The methods employed in these studies, and the results obtained are presented in the following section.

AGGLUTINATION TESTS

Materials and methods

Bacterial suspensions for agglutination tests were prepared from 18-hour broth cultures of streptococcus MG. The bacterial cells were washed 3 times in 0.85 per cent NaCl and suspended in sufficient saline to give a turbidity approximating No. 5 in the McFarland scale. The streptococci were killed by heating at 65° C. for 1 hour. Mergiolate, in a final concentration of 1:10,000, was added as a preservative.

Serial 2-fold dilutions of unheated serum were made in saline. Each serum dilution was then mixed with an equal volume of streptococcal suspension. The final dilutions of serum tested ranged from 1:10 to 1:320. Dilutions lower than 1:10 were not used in routine tests because of the frequent occurrence of non-specific agglutination of streptococcus MG in 1:2 or 1:4 dilutions of either normal human or rabbit serum. The tubes containing the serum and the streptococcal suspension were placed in a water-bath at 37° C. for 2 hours, followed by 18 hours in the ice-bath at 4° C. They were then again placed in the water-bath at 37° C. for 2 hours, after which the tubes were shaken and readings of the degree of agglutination were made. The final period of 2 hours at 37° C. was found to be of importance since non-specific agglutination of streptococcus MG occasionally occurred at icebox temperatures. Such agglutination disappeared when the mixtures were brought to 37° C.

Sera were not heated at 56° C., because it was found that the agglutination titer of convalescent serum was sometimes reduced by heating at this temperature.

In estimating the degree of agglutination, the following standards were adopted. A designation of 4+ was assigned to tubes in which agglutination was complete, with a solid plaque or disc of bacteria and a clear supernatant fluid. Agglutination with large clumps and a clear supernatant fluid, but without complete settling of the bacteria to the base of the tube, was designated as 3+. Agglutination with incomplete clearing of the supernatant fluid was designated as 2+. Agglutination with turbid fluid and with particles visible to the unaided eye, was designated as 1+. Agglutination which required the use of a hand lens for visualization was designated as \pm . The agglutination titer was taken as the highest dilution of serum in which reactions of 1+ or more were observed.

TABLE II

Results of agglutination tests against streptococcus MG with acute-phase and convalescent serum in primary atypical pneumonia

Case number	Day of disease	Dilution of serum					
		1:10	1:20	1:40	1:80	1:160	1:320
284	6	0*	0	0	0	0	0
	8	0	0	0	0	0	0
	14	4	4	4	4	4	3
	20	4	4	4	4	4	3
	29	4	4	4	4	3	0
	57	4	3	1	0	0	0
286	8	0	0	0	0	0	0
	10	0	0	0	0	0	0
	16	4	1	0	0	0	0
	32	4	4	4	4	2	0
	42	4	4	4	2	0	0
	54	4	2	0	0	0	0

* Figures refer to degree of agglutination
 4 = complete agglutination with disc formation
 1 = slight agglutination
 0 = no agglutination

The results of agglutination tests against streptococcus MG with serum specimens obtained from 2 selected patients at various periods during the course of primary atypical pneumonia are shown in Table II. It will be noted that, in both instances, agglutination did not occur with sera obtained early in the course of the illness, whereas marked agglutination took place with sera obtained later. With sera from one patient, the agglutination titer was found to be 1:320 on both the 14th day and 20th day after onset,

1:160 on the 29th day, and 1:40 on the 57th day. With sera from the other patient, the agglutination titer was 1:20 on the 16th day, 1:160 on the 32nd day, 1:80 on the 42nd day, and 1:20 on the 54th day after onset. As is indicated by the results shown in Table II, the agglutination produced by the convalescent serum of these patients consisted of large plaque-like discs of bacteria with a clear supernatant fluid. Vigorous shaking of the tubes caused some breaking up of the larger clumps but obvious agglutination persisted even after prolonged shaking. The gross appearance of the agglutinated bacteria was indistinguishable from that which was observed with this microorganism in the presence of anti-streptococcus MG rabbit serum.

The results of agglutination tests against streptococcus MG with acute-phase and convalescent sera from 193 patients with primary atypical pneumonia are summarized in Table III. It will be seen that of 158 acute-phase sera which were obtained during the first week of the disease, only 5 were capable of agglutinating this microorganism. In contrast, 130 (67.4 per cent) of a total of 193 convalescent sera agglutinated streptococcus MG in serum dilutions ranging from 1:10 to 1:320. It will be noted that the convalescent sera from 54 patients (28.4 per cent) of the entire group, possessed an agglutination titer of only 1:10. Each of these sera was re-tested, with similar results. The significance of agglutination titers of 1:10 is obviously open to

TABLE III

Results of agglutination tests with streptococcus MG and the serum of human beings

Diagnosis	Serum	Number of patients	Agglutination titer							Total number positive	Percentage of positive
			<1:10	1:10	1:20	1:40	1:80	1:160	1:320		
Primary atypical pneumonia	Acute	158	153	3	1	1	0	0	0	5	3.2
	Conv.	193	63	54	35	19	16	4	2	130	67.4
Acute respiratory infection without pneumonia	Acute	36	36	0	0	0	0	0	0	0	0
	Conv.	36	31	5	0	0	0	0	0	5	13.9
Other acute infectious diseases	Acute	83	83	0	0	0	0	0	0	0	0
	Conv.	84	82	0	0	1	1	0	0	2	2.4
Normal persons		75	75	0	0	0	0	0	0	0	0
Normal persons (Training Station)		246	210	29	7	0	0	0	0	36	14.6
Vaccinated persons (Strep. MG)	Before	11	11	0	0	0	0	0	0	0	0
	After	11	0	0	3	3	2	3	0	11	100.0

question, particularly since serum dilutions lower than 1:10 were not employed as a routine. Convalescent sera from 76 patients (39 per cent) possessed agglutination titers of 1:20 or more, and convalescent sera from 41 patients (21 per cent) had agglutination titers of 1:40 or more. As is shown in the accompanying paper (4), there appeared to be a positive correlation between the severity of the illness and the agglutination titer of the convalescent serum for streptococcus MG.

In Table III, the results of similar agglutination tests with sera from patients with other diseases are also shown. It will be seen that the convalescent sera of 5 patients with acute respiratory infections without pneumonia, so-called "catarrhal fever," agglutinated streptococcus MG at serum dilutions of 1:10. None of the sera from 31 other patients with a similar illness agglutinated this microorganism. Negative results were obtained with both acute-phase and convalescent sera from 82 patients with other diseases, including psittacosis (11 patients), pneumococcal pneumonia (30 patients), influenza A (21 patients), rheumatic fever (6 patients), early pulmonary tuberculosis (4 patients), and scarlet fever (10 patients). The serum of one patient with subacute bacterial endocarditis and streptococcus viridans bacteremia possessed an agglutination titer of 1:80 and the serum of another patient with empyema due to a Group F beta-hemolytic streptococcus had an agglutination titer of 1:40. None of the sera of 75 normal persons, who had no history of recent respiratory infection, were capable of agglutinating streptococcus MG. In agglutination tests with sera from 246 persons living at close quarters in a naval training station, 36 sera (14.6 per cent) showed agglutination titers of 1:20 or less. The sera from this latter group were obtained at a time when an outbreak of acute upper respiratory infections was in progress, and it is probable that many persons in the group had either been exposed or were recovering from such infections.

The results of agglutination tests with sera from 11 human beings who were given injections of either the capsular polysaccharide or heat-killed suspensions of streptococcus MG are also shown in Table III. Agglutinins were not

demonstrable in the serum of these 11 persons before vaccination. Following vaccination with a suspension of heat-killed streptococci, 2 persons developed agglutinins in titers of 1:20 and 1:40, respectively. Nine persons, who were injected with the capsular polysaccharide, developed agglutinins in titers ranging from 1:20 to 1:160. The agglutinins were demonstrable in serum obtained 8 days after vaccination, and were still present in undiminished titer 7 months later. It will be seen that the agglutination titers observed with sera from vaccinated persons were comparable to those obtained with the convalescent sera of patients with primary atypical pneumonia. The type of agglutination produced by the former sera was indistinguishable from that produced by the latter sera.

Agglutinins for streptococcus MG in the sera of patients with primary atypical pneumonia usually appeared between the second and fourth weeks of the disease and reached maximum titers during this period. In most cases, the agglutination titer began to diminish during the fifth and sixth weeks, and in some instances agglutinins were no longer detectable at this time. However, the sera of certain patients still possessed significant titers as late as nine weeks after onset. The variability in the time of appearance and disappearance of agglutinins for streptococcus MG was such that it was found advisable to obtain specimens of serum at weekly intervals from each patient, whenever this was possible.

The specificity of the positive agglutination reactions observed with convalescent serum was investigated by testing selected samples of strongly positive serum against suspensions prepared from cultures of other varieties of bacteria. These included *Hemophilus influenzae*, *Staphylococcus aureus*, pneumococci, and Group A hemolytic streptococci. In addition, 18 strains of other non-hemolytic streptococci which had been shown to be negative in the quellung test with anti-streptococcus MG rabbit serum were used. None of these various bacterial species was agglutinated by selected convalescent sera from patients with primary atypical pneumonia.

It has already been mentioned that an antigenic relationship exists between streptococcus MG and *Str. salicarius*, type I, which is ap-

parently due to antigenic similarities in the capsular polysaccharides of these two microorganisms (3). It was therefore of interest to determine whether suspensions of *Str. salivarius*, type I, were also agglutinated by convalescent sera. Acute-phase and convalescent sera from 99 patients with primary atypical pneumonia, each of whom was found to have developed agglutinins for streptococcus MG, were tested against suspensions prepared from cultures of *Str. salivarius*, type I, and *Str. salivarius*, type II.⁷ It was found that the convalescent sera of 6 of these 99 patients also were capable of agglutinating *Str. salivarius*, type I, and that the convalescent sera of 3 other patients agglutinated *Str. salivarius*, type II. With sera from the remaining 90 patients, agglutination did not occur in tests against either type I or type II *Str. salivarius*. Acute-phase and convalescent sera from 45 patients with primary atypical pneumonia, no one of whom developed demonstrable agglutinins against streptococcus MG, were also tested against suspensions of *Str. salivarius*, type I, and *Str. salivarius*, type II. It was found that none of the sera from these 45 patients was capable of agglutinating either type of this microorganism.

An analysis of the increases in titer observed in agglutination tests against streptococcus MG with sera from patients with primary atypical pneumonia is shown in Table IV. In a group of 99 patients whose sera yielded positive results, and from whom 2 or more samples of serum were

obtained during the course of the disease, a 2-fold increase in the titer of the convalescent serum was demonstrated in 47 patients, a 4-fold increase in 25, an 8-fold increase in 13, a 16-fold increase in 8, a 32-fold increase in 2, and a 64-fold increase in 1. In 3 patients, agglutinins were present in the acute-phase serum and no increase in the titer of the convalescent serum was demonstrable.

Analyses of the increases in titer observed in agglutination tests against either *Str. salivarius*, type I or type II, with sera from this same group of 99 patients, are also shown in Table IV. It will be seen that in tests with *Str. salivarius*, type I, no increase in the titer of the convalescent sera of 93 patients was observed, while a 2-fold increase in titer was demonstrated in 3 patients, a 4-fold increase in 1, an 8-fold increase in 1, and a 32-fold increase in 1. It will also be noted that in tests with *Str. salivarius*, type II, no increase in the titer of the convalescent sera of 96 patients was observed, while 2-fold, 4-fold, and 8-fold increases, respectively, were demonstrated in 3 patients.

It has already been mentioned that non-encapsulated, so-called R variants of streptococcus MG were obtained by inducing dissociation of this microorganism. These R variants possessed antigens which were immunologically distinct from the capsular polysaccharide of streptococcus MG (3). When acute-phase and convalescent sera from selected patients with primary atypical pneumonia were tested, by the agglutination technique, against such R variants, it was found that the convalescent sera agglutinated the R variants whereas the acute-phase sera did not. The agglutination of R variants produced by convalescent sera was distinctly different from the agglutination of encapsulated streptococcus MG produced by the same sera. Agglutination of R variants was characterized by the formation of very small bacterial clumps which were easily dispersed by gentle shaking. It is noteworthy that the agglutination of R variants produced by the serum of rabbits, immunized with either streptococcus MG or its R variant, was similar in all respects to that produced by the serum of patients convalescent from primary atypical pneumonia. These findings suggested that convalescent sera possessed

TABLE IV

Comparison of agglutination titers of acute-phase and convalescent sera of patients with primary atypical pneumonia

Streptococcal suspension	Number of patients	Increase in titer of convalescent serum						
		0×	2×	4×	8×	16×	32×	64×
Streptococcus MG	99	3*	47	25	13	8	2	1
<i>Streptococcus salivarius</i> , type I	99	93	3	1	1	0	1	0
<i>Streptococcus salivarius</i> , type II	99	96	1	1	1	0	0	0

* Figures refer to number of patients who showed designated increase in titer.

⁷ The strains of *Str. salivarius*, type I and type II, were obtained through the courtesy of Dr. J. M. Sherman of Cornell University School of Agriculture, Ithaca, N. Y.

STREPTOCOCCUS MG AND ATYPICAL PNEUMONIA

225

TABLE V
Results of agglutination tests with streptococcus MG and its R variant

MG AND ATYPICAL PNEUMONIA													
TABLE V													
Results of agglutination tests with streptococcus MG and its R variant													
Serum	Immunized with	Streptococcal suspension	Serum dilution (reciprocal)										
			10	20	40	80	160	320	640	1280	2560	5000	
Rabbit	Normal	MG	0	0	0	0	0	0	—	—	—	—	—
	Streptococcus MG	MG	+	+	+	+	+	+	—	—	—	—	—
	R variant of streptococcus MG	MG	+	+	+	+	+	+	±	3	1	0	0
Human	Normal	MG	0	0	0	0	0	0	0	0	0	0	0
	Streptococcus MG	MG	+	+	1	±	0	—	—	—	—	—	—
	Capsular polysaccharide of streptococcus MG	MG	4	4	4	4	2	0	—	—	—	—	—
	Acute phase serum	MG	0	0	0	0	0	—	—	—	—	—	—
	Convalescent phase serum	MG	4	4	4	4	0	—	—	—	—	—	—
Patient with primary atypical pneumonia		MG	0	0	0	0	0	—	—	—	—	—	—
		MG	+	+	+	+	+	—	—	—	—	—	—

MG = encapsulated streptococcus MG

R = R variant of streptococcus MG

antibodies specifically directed against at least the

separate and distinct antigenic components of

streptococcus MG. Further

this interpretation

MG = encapsulated streptococcus MG
R = R variant of streptococcus MG

antibodies specifically directed against at least 2 separate and distinct antigenic components of streptococcus MG. Further evidence supporting this interpretation was obtained from immunization experiments with streptococcus MG, its capsular polysaccharide, and an induced R variant in rabbits and in human beings. The results of certain experiments bearing upon this point are summarized in Table V. It will be seen that when either rabbits or human beings were immunized with encapsulated streptococcus MG, they developed agglutinins for both the R variant and the encapsulated form. When rabbits were immunized with the R variant, they developed agglutinins against the R variant but not against the encapsulated streptococcus. When human beings were immunized with the capsular polysaccharide, they developed agglutinins against the encapsulated streptococcus, but not against the R variant. It would therefore appear that the serum of patients convalescent from primary atypical pneumonia reacted in agglutination tests against streptococcus MG and its R variant in a manner analogous to

the serum of rabbits or human beings immunized with encapsulated streptococcus MG. It appears evident that such convalescent sera reacted differently in these tests from the sera of human beings, immunized only with the capsular polysaccharide, or the serum of rabbits, immunized only with the R variant.

PRECIPITATION TESTS

The capsular polysaccharide of streptococcus MG was employed in precipitation tests, using the capillary tube method (12), with the acute-phase and convalescent sera of 16 patients with primary atypical pneumonia. Similar tests were also made with the sera of 10 normal persons, before and after the intradermal injection of this polysaccharide.

The methods used for the extraction and purification of the capsular polysaccharide of streptococcus MG have been described in detail elsewhere (3). When the serum of rabbits immunized with streptococcus MG was tested against the capsular polysaccharide, visible precipitation occurred with the polysaccharide in

dilutions as high as 1:1,000,000. For tests with human sera, a 1:10,000 dilution of the polysaccharide was found to be satisfactory.

Convalescent sera from 11 of the 16 patients with primary atypical pneumonia produced visible precipitation with the capsular polysaccharide. None of the acute-phase sera from these patients yielded precipitates under identical conditions. Each of the sera from 10 normal persons was negative. Four weeks after the injection of the capsular polysaccharide, however, specific precipitins were demonstrable in the sera of 9 of these 10 persons.

Absorption tests were carried out with selected samples of convalescent serum, in order to determine whether the agglutination and precipitation reactions were produced by the same antibody. It was found that absorption of such serum with either a suspension of streptococcus MG or with its capsular polysaccharide resulted in the removal of both the agglutinins for the streptococcus and the precipitins reactive with the polysaccharide.

The precipitation test was found to be of limited usefulness with sera from patients with primary atypical pneumonia, since positive reactions were only obtained with sera which possessed relatively high agglutination titers. Moreover, positive precipitation tests were usually not obtained for as long a period during convalescence as were positive agglutination tests.

QUELLUNG TEST

Convalescent sera from 5 selected patients with primary atypical pneumonia, each of which had a high agglutination titer for streptococcus MG, were tested for their capacity to produce capsular swelling of this microorganism. A loopful of undiluted serum was mixed with a loopful of an 18-hour broth culture of streptococcus MG, and a loopful of 1 per cent methylene blue. The mixtures were then examined under the microscope for the presence of a positive quellung reaction. Definite quellung was produced by 2 different samples of convalescent serum, obtained from 1 patient during the second and third weeks of the disease. Each of these sera possessed an agglutination titer of 1:320. With the convalescent sera from 4 other patients,

which had lower agglutination titers, capsular swelling was not observed.

COMPLEMENT FIXATION TESTS

Complement fixation tests were performed with the acute-phase and convalescent sera of 12 selected patients with primary atypical pneumonia, using the capsular polysaccharide of streptococcus MG as an antigen. In other tests with this antigen, complement fixation had been shown to occur in high dilutions of anti-streptococcus MG rabbit serum. The method employed in these tests was the same as that used in tests with rabbit antiserum, and is described elsewhere (3).

It was found that the convalescent serum of 1 patient in a serum dilution of 1:32 produced complement fixation with the capsular polysaccharide of streptococcus MG. The convalescent sera of each of the other 11 patients were negative in this test, although some of these sera possessed relatively high agglutination titers and were positive in precipitation tests.

SKIN TESTS WITH THE CAPSULAR POLYSACCHARIDE OF STREPTOCOCCUS MG

The capsular polysaccharide of streptococcus MG was injected intradermally in 22 patients with primary atypical pneumonia, 14 patients with other acute infectious diseases, and 14 normal persons. Similar tests were also performed in 5 persons who had received an intradermal injection of the polysaccharide 2 weeks previously.

The skin test consisted of the intradermal injection in the anterior surface of the forearm, of 0.1 ml. of sterile saline containing 10 μ g. of polysaccharide. A control injection of saline was made in a corresponding area on the other arm.

In 7 of the 22 patients with primary atypical pneumonia who were given an intradermal injection of polysaccharide during the second or third week after onset of the disease, a definite response occurred at the site of inoculation. This reaction, usually consisted of an itching wheal surrounded by a zone of erythema. It appeared within 2 hours after the injection and persisted for 24 hours or longer. In 15 of these 22 patients, the reaction was negative. Negative

results were also obtained in 14 patients with other infectious diseases and in 14 normal persons.

In each of the 5 normal persons who had previously received an injection of the polysaccharide, positive skin reactions occurred which were similar in all respects to those observed in the patients convalescent from primary atypical pneumonia.

There appeared to be no direct correlation between the presence of circulating antibodies and positive skin reactions in the patients with primary atypical pneumonia, and it was not possible by means of serological tests to determine at what period during convalescence the reaction might become positive. Repeated skin tests in the same individual during the course of the disease produced results which could not be interpreted, since a single injection of polysaccharide stimulated the production of circulating antibodies against streptococcus MG and produced sensitization of the skin to a second injection 1 week later. The skin test was considered to be of limited practical usefulness, although the results obtained with it provided further evidence for the presence of specific antibodies against streptococcus MG in patients convalescent from primary atypical pneumonia.

PROPERTIES OF THE AGGLUTININ IN CONVALESCENT SERUM

Certain properties of the agglutinin for streptococcus MG in the convalescent sera of 2 patients with primary atypical pneumonia were investigated. The agglutination titer of these sera was not affected by alterations in pH over a range extending from pH 4.5 to 9.0. It was found by fractional precipitation with ammonium sulfate that the agglutinin was present solely in the water-insoluble portion of the globulin fraction of the serum. These properties are among those which characterize specific antibodies (13).

The agglutinin was found to be relatively thermolabile, as compared with certain other antibodies. Four-fold or greater reductions in the agglutination titers of certain sera were produced by heating the undiluted serum for 30 minutes at 56° C. and the agglutinin was almost entirely destroyed by heating at 60° C. for a similar period. It is of interest that the

same degree of thermolability was encountered with the agglutinins which developed in the serum of monkeys which had been inoculated intratracheally with streptococcus MG. Moreover, heating for 30 minutes at 56° C. reduced the agglutination titers of the sera of human beings who had received intradermal injections of the capsular polysaccharide of this microorganism.

It has been shown that the acute-phase sera of patients with primary atypical pneumonia contain an abnormal protein which reacts in precipitation tests with the C-polysaccharide of pneumococcus (4). This C-reactive protein is also known to be present in serum during the early stages of many other varieties of acute infectious disease (14). It was of importance to determine whether any relationship existed between this protein and the agglutinin for streptococcus MG in the serum of patients with primary atypical pneumonia. In a series of 18 patients, C-reactive protein was found to be present almost invariably in acute-phase serum, while agglutinins for streptococcus MG were not demonstrable in any of these sera. On the other hand, C-reactive protein was not present in any of the convalescent sera at the time when agglutinins for streptococcus MG had reached their maximum titers. These findings indicate that the agglutinin for streptococcus MG was distinct from the C-reactive protein.

It has also been shown that the convalescent sera of certain patients with primary atypical pneumonia are positive in the cold-hemagglutination test (6, 7), and in complement fixation tests with a variety of unrelated tissue antigens (5). Each of these non-specific serological phenomena was encountered with the sera of certain patients in this series (4). However, many convalescent sera were found to possess agglutinins for streptococcus MG in high titer while cold-hemagglutinins were not demonstrable. Furthermore, the sera of a few patients were capable of causing cold-hemagglutination in high titer, although they did not possess agglutinins for streptococcus MG. Similarly, a number of sera which possessed agglutinins for streptococcus MG failed to exhibit the property of non-specific complement fixation. These observations suggested that the agglutinin for

TABLE VI
Results of cross-absorption tests with the convalescent serum of a patient with primary atypical pneumonia

Serum	Test	Serum dilution				
		1:10	1:20	1:40	1:80	1:160
Unabsorbed	1) Agglutination of streptococcus MG	4	4	4	4	4
	2) Cold hemagglutination	4	4	2	2	0
	3) Complement fixation with mouse lung	4	4	1	0	0
Absorbed with strep. MG	1) Agglutination of streptococcus MG	0	0	0	0	0
	2) Cold hemagglutination	4	4	2	2	0
	3) Complement fixation with mouse lung	4	4	0	0	0
Absorbed with human RBC	1) Agglutination of streptococcus MG	4	4	4	4	3
	2) Cold hemagglutination	0	0	0	0	0
	3) Complement fixation with mouse lung	4	4	1	0	0
Absorbed with mouse lung	1) Agglutination of streptococcus MG	4	4	4	4	4
	2) Cold hemagglutination	0	0	0	0	0
	3) Complement fixation with mouse lung	0	0	0	0	0

streptococcus MG were independent of the other 2 serological phenomena. In order to determine whether any direct relationship existed, cross-absorption tests were carried out with several samples of convalescent serum which were positive in each of these 3 different tests. The results of a typical cross-absorption experiment are illustrated in Table VI. It will be seen that, before absorption, this serum produced agglutination of streptococcus MG in a titer of 1:160, cold-agglutination of human group 0 erythrocytes in a titer of 1:80, and complement fixation with normal mouse-lung antigen in a titer of 1:40. Following absorption of this serum with streptococcus MG, the agglutinins for this microorganism were completely removed while the titer of both cold-hemagglutination and non-specific complement fixation remained almost unchanged. Similarly, when the serum was absorbed with human group 0 erythrocytes, or with a suspension of mouse-lung tissue, the cold-hemagglutinins or the property of non-specific complement fixation were removed, while the agglutinin titer for streptococcus MG remained the same as before absorption. These results indicated that the agglutinin for streptococcus MG in convalescent serum was independent of the 2 non-specific serological phenomena mentioned above.

DISCUSSION

Non-hemolytic streptococci are known to be present commonly in the upper respiratory

tract of human beings, and are generally regarded as non-pathogenic saprophytes. Microorganisms belonging to this diverse group are encountered in cultures of the throats of many normal persons. The classification of non-hemolytic streptococci has not yet been completed although many workers have entered upon the problem. One distinct species of non-hemolytic streptococci, *Str. salivarius*, has been well characterized and can be distinguished readily from other non-hemolytic streptococci, both by biological and immunological tests (10). The so-called *Str. mitis* group appears to possess much less distinctive properties and probably cannot be considered to be a homogeneous species (10). The other varieties of non-hemolytic streptococci commonly present in the upper respiratory tract have not so far been classified satisfactorily.

The microorganism which has been referred to in this paper as streptococcus MG was readily differentiated from either *Str. salivarius* or the *Str. mitis* group. The strains of streptococcus MG which have been studied showed a sufficiently striking homogeneity as regards both their biological and immunological characteristics to warrant the conclusion that they constituted a distinct species and belonged to a single serological type.

Streptococcus MG was isolated from the lung tissues of 6 of a total of 8 patients who died of primary atypical pneumonia, and was not obtained from the lung tissues of 6 patients who

died of other causes. It was isolated from the sputum or throat swabs of 53 of a total of 97 patients with primary atypical pneumonia, and 20 of a total of 82 patients with other acute infectious diseases, as well as 7 of a total of 57 normal persons.

Evidence was obtained which indicated that in 4 of the lung tissues from fatal cases of primary atypical pneumonia, streptococcus MG was present in numbers of the order of 100,000 or more per gram of lung tissue.

It was found that 67 per cent of 193 patients with primary atypical pneumonia developed, during convalescence, antibodies directed against streptococcus MG. The available evidence indicates that the serological reactions obtained with this non-hemolytic streptococcus and the convalescent sera of patients with primary atypical pneumonia were due to the presence of specific antibodies and not to some non-specific property of these sera analogous to that responsible for cold-hemagglutination (6, 7) or complement fixation with various tissue antigens (5). With but relatively few exceptions, such antibodies were demonstrable only in the convalescent sera of these patients and not in the serum of patients with other infectious diseases. Positive reactions did not occur when convalescent sera possessing high agglutination titers for streptococcus MG were tested against a number of other bacterial species. *Str. salivarius*, type I, which has been shown to possess certain antigens in common with streptococcus MG, was agglutinated only by some 6 per cent of convalescent sera which were capable of agglutinating the latter microorganism. Moreover, it has been shown that the antibodies in the convalescent sera of patients with primary atypical pneumonia reacted with at least 2 separate and distinct antigenic components of streptococcus MG, namely, its capsular polysaccharide and one or more somatic antigens.

The significance of this non-hemolytic streptococcus in relation to primary atypical pneumonia is not yet clear. There appear to be a number of possible explanations which must be considered separately. First, it seems possible that the observed serological reactions might be the result of a coincidental antigenic relationship between this non-hemolytic streptococcus

and some other agent, perhaps a virus, which is itself the causative agent in primary atypical pneumonia. This possibility may be considered as analogous to the positive serological reactions obtained with members of the *B. proteus* group in various rickettsial diseases. There are, however, certain reasons for doubting the validity of this hypothesis. It would hardly account for the presence of this streptococcus in considerable numbers in the lungs of fatal cases of primary atypical pneumonia. Furthermore, it would be surprising if there were an antigenic relationship between 2 separate and antigenically distinct components of this bacterium and some other agent. In view of the presence in convalescent serum of antibodies directed against both the capsular polysaccharide and somatic antigens of streptococcus MG, such an assumption would be necessary.

Secondly, it seems possible that this non-hemolytic streptococcus might occupy the rôle of secondary invader in primary atypical pneumonia. In this regard, it is of interest that other investigators have called attention to the frequent occurrence of non-hemolytic streptococci in primary atypical pneumonia (15, 16). The possibility that these microorganisms may represent secondary invaders has been suggested (17, 18). An association between non-hemolytic streptococci and other clinical varieties of pneumonia has also been reported by a number of investigators (19 to 22). The possibility that streptococcus MG may be involved in primary atypical pneumonia as a secondary invader cannot be excluded. However, this term usually implies an accidental or random association between an infectious agent and a disease, rather than an association with such a relatively high proportion of cases as is indicated by the results of this study. It seems rather unlikely that an accidental association should occur with microorganisms which, in each instance, were members not only of a single species but also of one serological type.

Thirdly, it seems possible that this non-hemolytic streptococcus, either alone or in concert with some other infectious agent, might be primarily involved in the pathogenesis of primary atypical pneumonia. The available evidence is not sufficient to warrant the accept-

tance of this hypothesis at the present time, although the results of this study suggest paths for further exploration.

SUMMARY

Non-hemolytic streptococci, comprising a homogeneous group and belonging to a single immunological type, have been isolated from the lungs and sputa of patients with primary atypical pneumonia. Specific antibodies directed against this species of streptococcus have been demonstrated in the convalescent serum of patients with primary atypical pneumonia by means of a number of serological techniques. With few exceptions, such antibodies were not demonstrable in the serum of these patients during the acute phase of the disease, in the serum of patients with other diseases, nor in the serum of normal persons.

The significance of these results is not yet clear, but they suggest the possibility that this non-hemolytic streptococcus may, in some manner, be implicated in the pathogenesis of primary atypical pneumonia.

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A CLINICAL REPORT ON CASES OF PRIMARY ATYPICAL PNEUMONIA CAUSED BY A NEW VIRUS¹

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During the 10 years which have passed since the disease now designated as primary atypical pneumonia began to take form as a definite clinical entity, considerable progress has been made in studies of its clinical and epidemiological aspects (1). Progress in etiological studies, however, has been comparatively slow. The isolation of a number of infectious agents from single patients or small groups of patients (2 to 10) has made it clear that the clinical syndrome under discussion is of diverse etiology and that the human organism, like a variety of experimental animals, may show a somewhat similar clinical and pathological response when any one of a number of unrelated nonbacterial infectious agents is introduced into the lower respiratory tract. At the same time, there is no convincing evidence that the great majority of cases reported have been due to any of the viruses or rickettsiae isolated up to the present.

The discovery that an infectious agent in the sputum or lungs of patients with primary atypical pneumonia produced a pneumonic infiltration when inoculated intranasally into cotton rats (11) opened a new line of approach. Investigation was further facilitated by the subsequent demonstration that this agent, presumably a virus, multiplied when introduced from human material into the amniotic sac of the chick embryo and that suspensions of infected chick embryo tissues produced a similar pneumonic response in cotton rats and hamsters. The properties of this virus, which is apparently

not related to influenza virus (type A or B), psittacosis-like viruses, or the rickettsia of Q-fever, have been described elsewhere (12). It was found that serum taken early in the course of the disease failed to neutralize the virus, but that convalescent serum consistently protected the test animals. On the basis of this neutralization test, it has been possible to classify a group of cases of primary atypical pneumonia of a type which has, up to the present time, fallen into the category of unknown etiology.

The correlation of the clinical and pathological findings in the various types of primary atypical pneumonia with the responsible etiological agent constitutes an important problem at the present time. In a previous report (13), clinical, laboratory, and pathological data have been presented on a group of 10 patients with atypical pneumonia from whom psittacosis-like viruses were isolated. It was emphasized that infections due to those agents were comparatively rare and differed in certain important respects from those not due to psittacosis-like viruses. The purpose of this paper is to present similar data on another group of cases of primary atypical pneumonia, together with evidence that they were caused by this new atypical pneumonia virus. Criteria of proof of such an etiological relationship are (1) demonstration of virus in lung or sputum during the course of illness by animal inoculation or by adaptation to chick embryos, (2) demonstration of an increase in neutralizing antibody during or after recovery from the illness, or preferably (3) both.

The 16 cases presented represent that form of primary atypical pneumonia which has occurred frequently in this area during the past 4 years, either as an epidemic or endemically. The group is somewhat weighted in favor of the more severe cases because those are more likely

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² Representing the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General of the United States Army.

investigated. It is of considerable interest that most of the patients in the group developed significant titers of cold agglutinins (14).

MATERIAL AND METHODS

Clinical material. The cases studied, with one exception, were selected from a larger group of approximately 100 cases which occurred in students at the University of California in Berkeley and at the University of California Medical Center in San Francisco during the period from July 1942 to March 1944. All the patients were between 19 and 27 years of age. Ten of them were males. The single fatal case occurred at Letterman General Hospital.

Isolation of virus. Specimens of sputum or lung were ground with alundum, diluted with broth to 10 or 20 per cent suspensions, lightly centrifuged, and inoculated intranasally in 0.4 ml. amounts into cotton rats under ether anesthesia. Animals were sacrificed 7 to 14 days later, and the result was interpreted as positive when a definite area of pulmonary consolidation was present. Attempts to produce similar consolidation consistently by passage of lungs have, to date, failed or have been masked by the acquisition of other viruses, presumably of animal origin.

For this reason, isolation and passage in the amniotic sac of chick embryos has been used for most purposes. One strain was adapted to eggs from a sterile specimen of human lung without filtration. Two other strains were derived from sputum specimens which had been filtered through collodion membranes of 600 m μ average pore

diameter. Passage has been made in 11- or 12-day-old embryos at 5-day intervals, amniotic membrane, trachea, and lungs being ground together for passage material. Presence of virus was detected by inoculating suspensions of these tissues intranasally into hamsters or cotton rats. Pulmonary consolidation like that following sputum inoculation was observed if virus was present in sufficient amounts.

Neutralization tests. Suspensions of chick embryo tissues, previously tested for ability to produce pulmonary consolidation in test animals, were mixed with an equal volume of serum. Acute and convalescent sera were inactivated and diluted with an equal volume of horse serum before mixing with the virus suspension, the test serum dilution being 1:4. After 20 minutes' incubation at room temperature, these suspensions were inoculated intranasally into hamsters or cotton rats. Four animals were tested with each suspension. Hamsters were sacrificed at 7 days and cotton rats at 12 to 14 days. The results were expressed as a fraction, the numerator indicating the number of animals showing pulmonary consolidation, and the denominator, the total number tested. A mixture of the original virus suspension with an equal amount of horse serum served as virus control, while a suspension of normal amniotic membrane, trachea, and lungs and an equal amount of horse serum served as animal control. Because the virus is labile, animals receiving the convalescent serum suspension were always inoculated before those receiving the acute serum suspension. Results of neutralization tests, together with certain clinical and laboratory findings, are presented in Table I.

TABLE I

Summary of certain clinical and laboratory findings, cold agglutination tests, results of inoculation of sputum into cotton rats, and neutralization tests in 16 cases of primary atypical pneumonia

Case	Highest temp.	Duration of fever	White blood cell range	Lobes involved	Cold agglutinin titers		Result of inoculation of sputum into cotton rats	Neutralization tests	
					Day bled	Titer		Acute serum	Convalescent serum
Ir	° F.	days							
Ki	104	9	5,800 to 9,600	RLL	5*, 37†		2/2	3/4	0/4
Pa	103.6	14	8,800 to 9,550	RML, RLL	7*, 43†		2/4	2/8	0/8
Yo	101	12	9,300	RML	7*, 13, 27†	10, 80, 40	1/2	6/7	0/7
Sn	103.6	9	8,450	RLL, LLL	3*, 11, 37†	<10, 40, 40	4/6	2/4	0/4
Mu	104	9	5,500 to 12,400	RUL, RLL	4*, 16, 26, 80†	<10, 2,560, 640, 320	7/12	3/8	0/8
Bu	104	10	4,950	RLL	6*, 9, 16†	<10, 80, 320	2/2	2/4	0/4†
So	104	14	5,100 to 8,300	RUL	4*, 12, 18†	<10, 40, 1,280	6/9	6/7	0/7
Do	104.8	24	7,750 to 37,600	RLL, RUL, LLL, LUL	4*, 12, 25†, 46†	<10, 80, 160, 20	5/9	6/11	0/11†
Hu	102	8	8,200 to 13,200	LLL	7*, 12, 19, 27†	20, 320, 160, 80		3/3	0/3†
Kr	101.8	16	10,000 to 7,900	LLL, RLL	8*, 14, 21, 28†	5, 40, 160, 160	6/10	4/4	0/4†
Mur	104.8	19	7,650 to 11,600	RLL, LLL	7*, 14, 21, 28†	<10, 320, 160, 80		1/4	0/4
Ta	101.4	7	6,600 to 10,700	LLL	7*, 16†, 76	<10, 160, 10		3/8	0/8†
Pg	101.8	8	9,100 to 8,900	RLL	6*, 15†, 60	<10, 80, 10		3/4	1/4
De	103.4	16	8,200 to 13,100	RLL	4*, 10, 19, 31†	<10, 10, 80, 80	1/4	4/4§	0/4
	101	8	8,200	RML	5*, 11, 21, 50†	<10, 80, 80, 40	8/27	3/4§	0/4
	105.4	12	8,000 to 22,000	LLL, LUL, RLL, RML, RUL					

* Acute serum. † Convalescent serum specimen used in neutralization tests. ‡ Serum shown by subsequent test to neutralize at dilution of 1:16 or more. § Neutralization test done in chick embryos. || Died.

In the last three columns numerator indicates number of positive animals; denominator the total number tested.

CLINICAL PICTURE

The onset of the disease was, in most instances, gradual. Presenting symptoms most frequently noted were malaise, cough, feverishness, headache, and moderate aching. Chilly sensations were common, but true chills infrequent. Sore throat, if present, was not severe (15). Pleuritic pain was not noted, though chest pain, either retrosternal in location or felt along the costal margin, was present in several instances. Cough was often troublesome and persistent. Sputum was scanty, usually tenacious, greenish in color, and occasionally streaked with bright red blood. On physical examination, the patient did not appear severely ill unless considerable areas of lung were involved. Slight dulness and scattered moist râles were often the only findings on examination of the chest. Later in the course of the disease coarse râles were heard and signs of consolidation sometimes appeared.

Admission white blood cell counts ranged from 4,950 to 10,000. As the illness progressed, particularly when it was severe, the white blood cell count tended to rise. Thus, in the single fatal case (De) it rose to 22,000 before death, and in another extremely severe case (Bu) a count of 37,500 was recorded at the height of the disease. Sputum cultures revealed only the usual pharyngeal organisms. Stained smears showed few bacteria and a relatively sparse cellular exudate in which mononuclear cells were common. X-rays of the lungs showed small or extensive infiltrations. Lateral films were often of value in disclosing minimal lesions. Not infrequently, the areas of pneumonia continued to spread for several days or new areas were noted, but the migratory type of pneumonia was uncommon.

The temperature curve was either very irregular or fairly sustained, and usually fell by lysis. Most patients began to show clinical improvement and clearing of x-ray findings within 10 days of onset. At no time did they appear very ill. In others, however, the pneumonic process continued to spread and the patients became desperately ill and showed intense dyspnea and cyanosis. The one fatal case was remarkable in that within 11 days the pneumonia had involved most of one lung and more than half of the other. Herpes labialis was noted in

patients with high fever. The spleen was palpable in one patient (Yo) during the first week of illness. Disturbance of cerebral functions was infrequent even in those patients who were most seriously ill. Convalescence was slow, many patients complaining of weakness, dyspnea on exertion, and persistent cough. No other sequelae have been observed.

CASE SUMMARIES

Three cases are presented in detail. The first, a comparatively mild one, was selected as an example of the type of case most frequently seen. The second case, which was extremely severe, and the third, which terminated fatally, represent less common types, but present a number of points of unusual interest. Clinical data on these 3 and 13 other cases are presented in Table I.

Case 1. Gi, a male, aged 22, awoke on March 21, 1943, with a tickling sensation in his throat. That evening he felt feverish and complained of generalized aching and malaise. Next day these symptoms were more pronounced and a troublesome cough had developed which produced small amounts of tenacious greenish sputum. It is of interest that the patient's brother, with whom he was living, had previously suffered a similar attack of primary atypical pneumonia, beginning on March 1, 1943, and had shown a rise in cold agglutination titer from less than 10 during the acute phase to 40 during convalescence. The 2 brothers were in contact with each other until March 9.

On admission to the University of California Hospital on March 22, the patient's temperature was 103.6° F., pulse 100, respirations 20, and blood pressure 108/80. He did not appear acutely ill, but complained of severe generalized headache, retro-orbital soreness, and occasional pain in his right ear. His skin was hot and dry, his nose moderately congested; but no other symptoms were noted.

Course: On March 24, slight dulness and a few crackling râles were noted at the base of the right lung. These became more prominent during the following days. The temperature remained elevated until March 29, with daily peaks of 102.2° or more, and fell by lysis. Throughout this period, the patient felt and looked comfortable and continued to eat well. Headache and cough were the most troublesome symptoms. Recovery was fairly rapid.

Laboratory findings: On March 23, the blood count showed 5,000,000 red blood cells (hemoglobin 14 grams) and 8,450 white blood cells with 70 per cent polymorphonuclears (F.N.F. 34/36). Sputum examination was negative for pneumococci and acid-fast bacilli. Chest x-ray on March 23 showed a small patch of pneumonia in the right lower lung in the cardiophrenic angle. On March 24, this area had spread and involved a good deal of the right lower lobe and there was evidence of a small area of consolidation

middle of the left lung. On April 22, the lungs appeared normal.

Suspensions of sputum collected on the fourth day of disease produced pulmonary lesions in 4 of 6 cotton rats. Cold agglutination titers (as shown in Table I) of serum specimens taken on the third, eleventh, and thirty-seventh days of disease were less than 10, 40, and 40, respectively.

Case 2. Bu, a female, aged 20, noted aching and malaise on April 26, 1943. During the following 2 days, these symptoms became more troublesome, and in addition she complained of cough, feverishness, restlessness, and anorexia. Slight wheezing with respiration was noticed. She was admitted to Cowell Memorial Hospital on April 29.

Physical examination: The patient's temperature was 100.2° F., pulse 84, respirations 22. She did not appear acutely ill. Cough was frequent and produced moderate amounts of tenacious, yellowish sputum. Conjunctivae and pharynx were injected. Fine and coarse râles were heard over the right lower lobe as far anteriorly as the axillary line.

Course: The high, spiking temperature curve is reproduced in Figure 1. On May 1, bronchial breathing was heard over the right lower lobe and the patient was placed in an oxygen tent. On May 5, dulness was noted over the right lower lobe and bronchial breathing was heard over most of the right lung and over the upper half of the left lung. On May 6, respirations were wheezing in character. By May 7, respirations had risen to 38, cyanosis was marked, and the patient appeared seriously ill. On May 9, 200 ml. of pooled plasma from 3 patients recently convalescent from primary atypical pneumonia, who had shown significant cold agglutinin titers, were given intravenously. On the next day, the patient appeared somewhat more alert and 300 ml. more of the same pool of plasma were given. One hour later, her pulse had risen to 140 and she was apprehensive and sweating

profusely. That evening, the respiratory rate was 50, pulse 156, and the patient appeared moribund. She remained in an extremely critical state throughout the next day, and then slowly improved. Her temperature meanwhile had fallen and subsequently did not exceed 101°, though another 10 days passed before it returned to normal. Convalescence was slow, but at last report there were no sequelae. Sulfadiazine was given from May 3 to May 10, the blood level on May 4 being 7.1 mgm. per cent. No effect on the course of the illness was seen.

Laboratory findings: Admission white blood cell count was 7,750. As the illness progressed, the count rose steadily. On May 9, it was 17,200, with polymorphonuclears constituting 86 per cent (F./N.F. 56/30). On May 12, it was 37,600. It dropped to 24,400 on May 15, to 19,700 on May 20, and to 9,200 on May 30. Sputum cultures showed a growth of *Staphylococcus aureus* and alpha streptococci, but were negative for pneumococci, beta streptococci, and acid-fast bacilli.

Chest x-ray on April 29 showed a fairly extensive pneumonic area at the base of the right lung, lobar in character, and mottling near the left hilum. On May 6, films showed an extensive pneumonia involving much of the right lung and the middle portion of the left lung. On June 3, there was much clearing, but slight residual infiltration on both sides. On June 11, clearing was more complete, but an area of increased density, which suggested fibrosis, remained at the left apex.

Sputum collected on April 29 and May 6 was tested in cotton rats. The results with both specimens were positive, 5 out of 9 animals showing pulmonary consolidation. Later, this material was passed through a collodion membrane of 600 m μ pore diameter, and the virus was adapted to chick embryos. This strain has been carried through 25 passages and is neutralizable by the convalescent sera of this and other patients with atypical pneu-

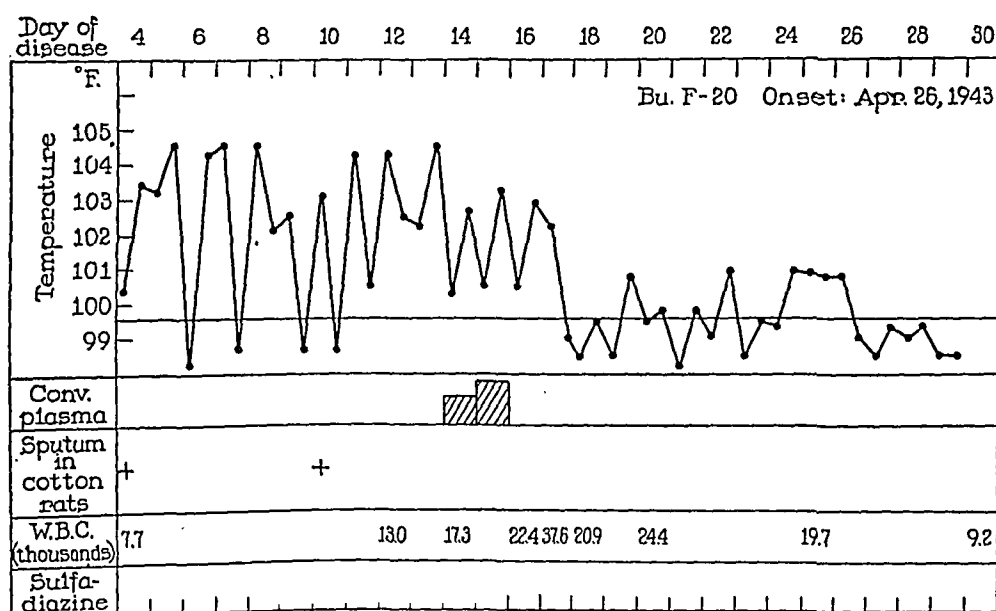


FIG. 1. TEMPERATURE RECORD OF PATIENT (BU) WHOSE ILLNESS WAS UNUSUALLY SEVERE

monia. The acute serum does not neutralize the virus. The results of serial cold agglutination tests are recorded in Table I.

Case 3.¹ De, a male, aged 26, was admitted to Letterman General Hospital on November 11, 1942, with a history of an illness of 4 days' duration. Complaints during this period were coryza, sore throat, cough, feverishness, malaise, generalized muscular pains, and anorexia. Symptoms had become markedly worse on the day of admission.

Physical examination: The patient's skin was hot and dry. The right nostril was obstructed and the pharynx injected. Respiratory movements were equal and shallow because deeper respiration incited cough and pain in chest. Slight dulness to percussion was noted at the bases of both lungs, and many fine moist râles were heard throughout the lungs, particularly at the bases. Blood pressure was 118/50. Examination of the heart showed it to be normal.

Course: Fever remained high, with peaks reaching 105° F. The patient soon became markedly dyspneic and cyanotic and was placed in an oxygen tent. Signs of pneumonia became more prominent over the right upper lobe and the whole left side. The cough was troublesome, but virtually no sputum was raised. Sulfadiazine was given throughout hospitalization in doses of 1 gram every 4 hours and an adequate blood level was obtained, but no effect was apparent. Dyspnea became progressively worse, and the patient died on November 17.

Laboratory findings: Admission blood count showed 3,770,000 red blood cells and 8,000 white blood cells, with normal distribution. Subsequently, the white blood cells rose to 11,000, to 16,000, and finally to 22,000 on November 16. Urinalysis showed albumin (++). Throat swabs and a single sputum examination failed to show pneumococci. Chest x-ray taken on November 11 revealed an extensive bronchopneumonia in the left lower lobe. On November 15, x-ray showed an extremely extensive bronchopneumonia involving both lobes on the left and parts of all three right lobes.

Autopsy findings: Post-mortem examination was made within 1 hour after death. Intestines were markedly distended. A slight excess of blood-tinged fluid was found deep in the peritoneal cavity. Both pleural cavities contained about 200 ml. of slightly blood-tinged fluid. The pericardial cavities contained about 200 ml. of pale straw-colored fluid.

The right lung weighed 1,085 grams, the left 945 grams. The lungs were similar. On palpation, from apex to base, there were noted innumerable distinct and confluent soft and firm nodules, which varied from about 1 to 4 cm. in diameter. Lymph nodes at the hilum were somewhat enlarged, soft, and greyish between the areas of pigmentation. Bronchi were distended with yellow, purulent, frothy exudate. Beneath the pleura the nodular foci appeared as lighter or darker areas against the slate-grey

background. Except for a number of areas over the left lung, where dull and granular areas were covered with a pinkish-red exudate, the pleura was smooth and showed no exudate. On cut surface, the nodular areas could be seen as distinct or confluent, frequently poorly circumscribed, dry granular foci of irregular size and shape, which stood above the cut surface of the lung. The nodules were sometimes pink and duller than the surrounding tissue. Some were grey and many had a yellowish cast, as though slightly broken down. This pneumonic process extended from apex to base, involving all lobes.

The heart weighed 430 grams. The right auricle and ventricle were markedly dilated. The coronary arteries were smooth, and the lumens were patent. The myocardium was red and of good tone. The spleen weighed 250 grams. The cut surface showed a soft grumous red pulp, and the follicles were not observed. The brain weighed 1,640 grams. Cerebral vessels were markedly congested, particularly the pial vessels. Other organs showed nothing noteworthy.

Microscopic examination: All sections (Figure 2) taken from different portions of the two lungs showed a pneumonic reaction which might be classified as an atypical bronchopneumonia. Practically all combinations of cellular, fibrinous, and serous exudate could be found in the various alveoli, the exudate in some appearing fresh, while in others it seemed to be undergoing resolution. In some sections, the character of the pneumonia was that of a discrete bronchopneumonia. In other areas, the pneumonia was of the confluent bronchopneumonic type.

Certain features in the inflammatory reaction were of interest. In the first place, there was a good deal of fibrin present, much of which was distributed peripherally and appeared tightly adherent to the inner wall of the alveolus. In the second place, there were certain areas in which it seemed that the exudate was breaking down and abscesses were forming. In these areas, the exudate was cellular, often consisting predominantly of polymorphonuclear leukocytes. Necrosis of the alveolar walls had apparently occurred and the center of the pneumonic focus seemed to be undergoing liquefaction. A third point of interest was that in certain areas the exudate appeared to be undergoing organization, the process consisting almost exclusively of invasion by young fibroblasts with no proliferating capillaries observable. A fourth point of interest was the marked morphological change which had taken place in the epithelial lining of a few of the bronchi. Here the epithelial lining had changed wholly or in part to a stratified squamous type. The appearance of the epithelial lining of these bronchi was that of a pavement type of epithelium with loss of cilia, squamous cells on the surface, and pyknotic cells interspersed in the deeper layers. Mitotic figures were extremely numerous. In several sections showing strips of pleura, the pleura was practically always free of exudate.

The coronary arteries were normal. Section of pericardium revealed sparse fluid, congested and hyperemic vessels, some beingumpy and pale at base of section.

¹ This case is reported through the courtesy of the Medical and Laboratory Branches, Letterman General Hospital, from whom the clinical and pathological findings were obtained.

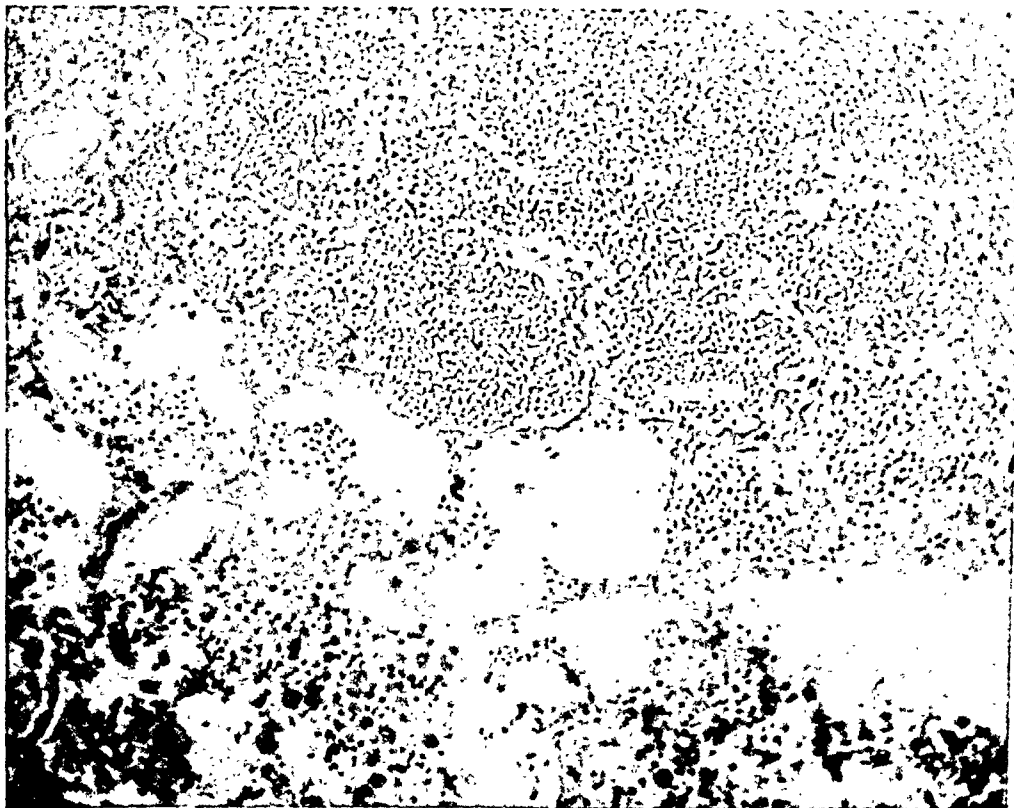


PLATE 1. Section from edge of a small discrete pneumonic focus, showing dense cellular exudate in alveoli in upper portion and comparatively sparse cellular exudate with numerous macrophages in lower portion. $\times 120$.

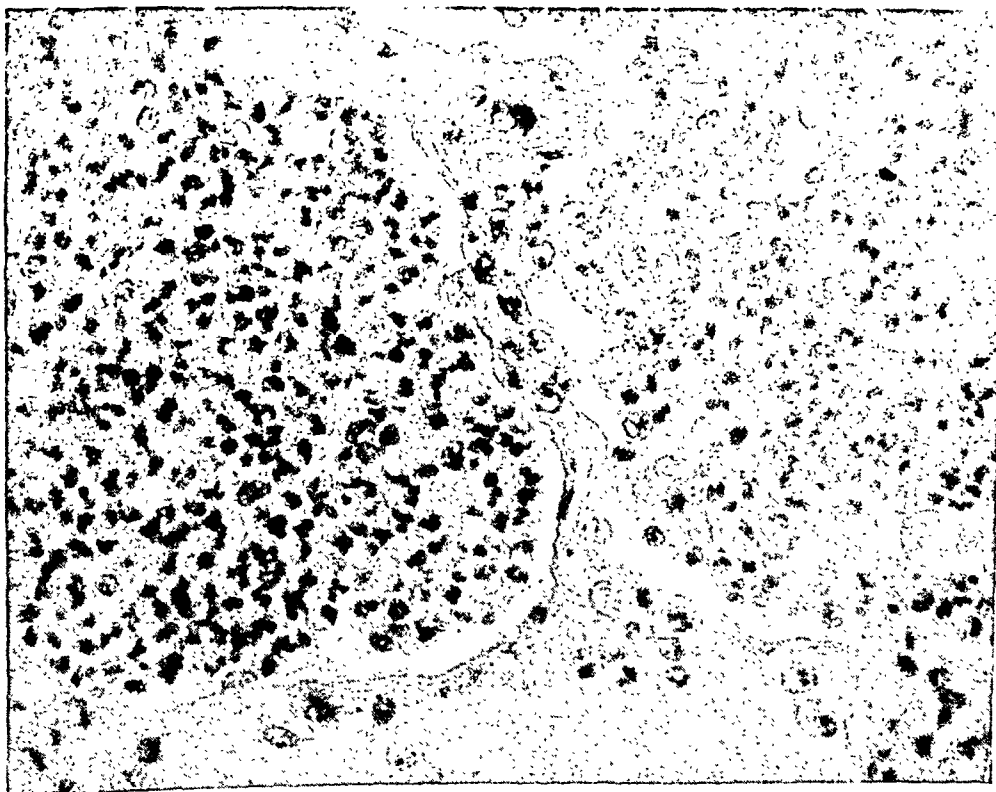


PLATE 2. High magnification of same section, showing composition of alveolar exudate. Small mononuclear and polymorphonuclear cells predominate and occasional macrophages are seen. The alveolar septa are not markedly thickened. $\times 400$.

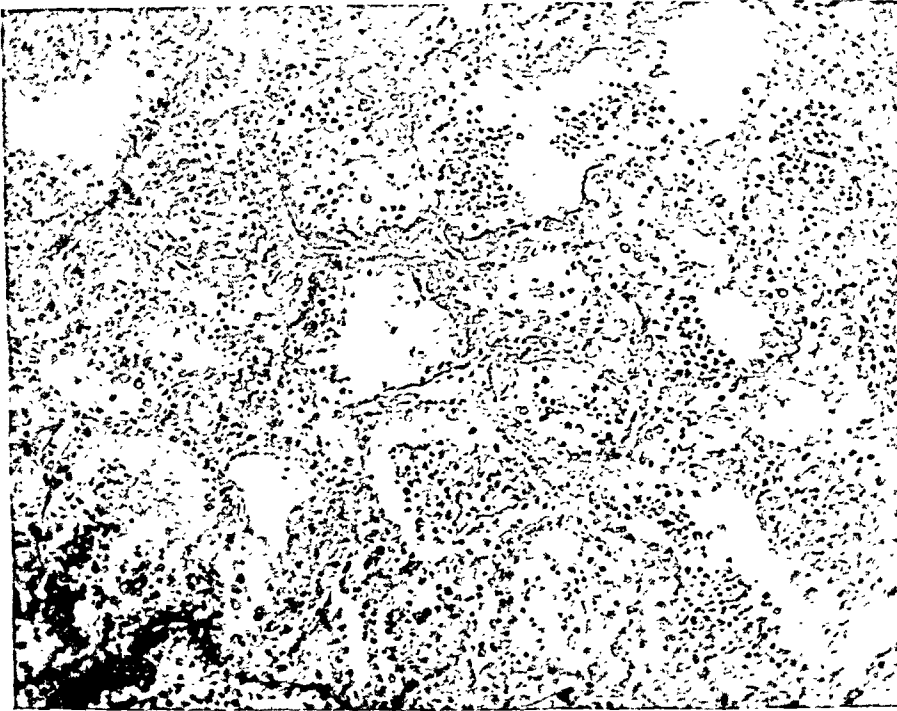


PLATE 3. Section from another area, showing fibrin distributed peripherally along some of the alveolar walls. The exudate in this area is less cellular. $\times 120$.

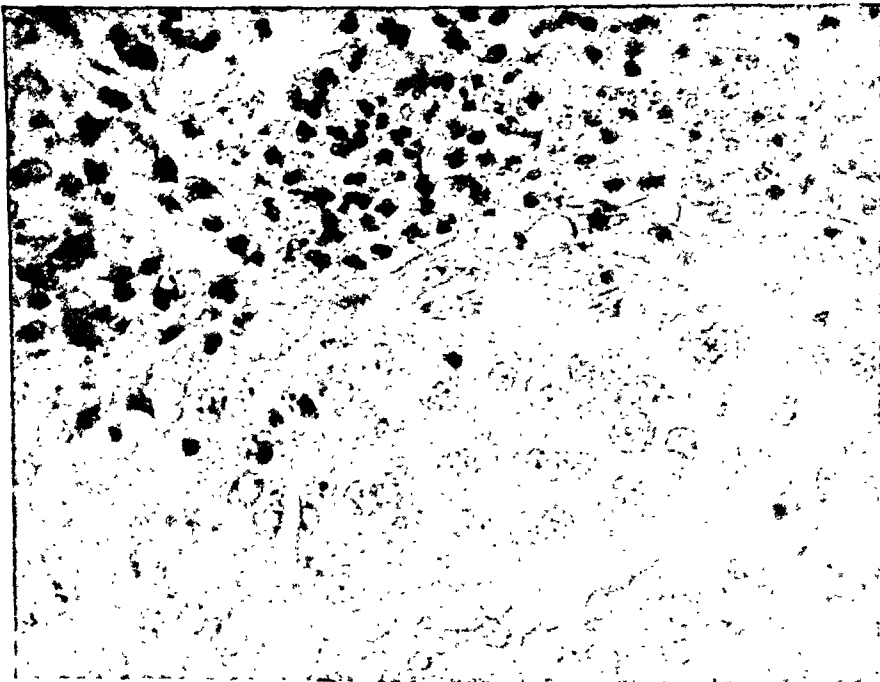


PLATE 4. Section showing metaplasia of the bronchial epithelium. The ciliated columnar epithelium has changed to an irregular stratified squamous type. $\times 400$.

FIG. 2. SECTIONS FROM LUNGS OF PATIENT (DE) WITH FATAL CASE OF PPRV. ATYPICAL PNEUMONIA. STAINED WITH HEMATOXYLIN AND EOSIN.

showing loss of striation. The small capillaries of the myocardium were markedly congested, and some contained numerous leukocytes. In one or two sections, leukocytes were present in the somewhat edematous interstitial tissue outside the capillary walls. There were, however, no definite areas of infarction and no specific rheumatic nodules. Sections from other organs showed nothing noteworthy.

Etiological studies: Cultures of lung tissues remained sterile after 72 hours. Impression smears showed no bacteria or elementary bodies. Suspensions tested intranasally in cotton rats produced pulmonary lesions in 8 of 27 animals. Without filtration, the virus in the human lung suspension was adapted to the amniotic sac of chick embryos and has been carried through 45 passages. This procedure was subsequently repeated. Suspensions of amniotic membrane, lungs, and trachea retain their infectivity for cotton rats and hamsters and are neutralized by convalescent serum from other persons who have had primary atypical pneumonia. The properties of the virus isolated from this patient have been described in detail in a separate publication (12). A cold agglutination test run with serum pipetted from the lung specimen showed a titer of less than 10.

DIFFERENTIAL DIAGNOSIS

An attempt to differentiate primary atypical pneumonia from other diseases is rendered difficult by the fact that several diseases of different etiology have been included under this heading. The observations which follow will therefore be restricted to the type of the disease prevalent in the area which the cases described in this report represent. While these cases present a fairly characteristic picture, the number of the diseases which may cause difficulty in diagnosis is very large. Thus, mild cases with slight or, perhaps, no pulmonary involvement may closely resemble certain nonbacterial upper respiratory infections, particularly influenza and other influenza-like diseases.

The more familiar form of the disease, with slight or extensive pulmonary involvement, must be distinguished on the one hand from bacterial and mycotic pneumonias, and on the other from pneumonias due to other viruses and rickettsiae. Pneumococcal lobar pneumonia presents several features very uncommon in primary atypical pneumonia, notably pleuritic pain, rusty sputum, and leukocytosis early in the disease. Bronchopneumonias due to pneumococci, streptococci, staphylococci, and other bacteria may cause more difficulty. Those primary atypical pneumonias restricted to upper lobes or presenting a

very diffuse pneumonia may closely resemble pulmonary or miliary tuberculosis. The picture of primary coccidioidal pneumonia may be very similar, and isolation of *Coccidioides immitis* and serological tests may be necessary for differential diagnosis (16). It is possible that the cold agglutination test may prove extremely useful in separating out the primary atypical pneumonias, but further substantiation of this point is needed.

The clinical differences between primary atypical pneumonia and pneumonias due to psittacosis-like viruses or Q-fever are sometimes too slight to permit differentiation, but suspicion may be directed toward the psittacosis group when a severely ill patient shows marked impairment of cerebral function and prominent gastro-intestinal symptoms, and appears gravely ill despite the fact that the area of lung involved is comparatively limited. A history of avian contact should be sought. Laboratory procedures, including isolation of virus and demonstration of an increase in antibody to the agent in question are usually required.

TREATMENT

Symptomatic therapy, directed chiefly toward relieving headache and cough, was used in all cases. Only in the most severe cases were sulfonamides employed, and in no instance was any response observed. Oxygen was administered to 6 patients and appeared to have a beneficial effect. One patient (Bu) received a pool of convalescent plasma from patients who had previously shown high cold agglutinin titers. Her temperature fell after the second transfusion, but her condition never appeared worse than during the following day. X-ray therapy was not tried.

DISCUSSION

The cases of primary atypical pneumonia studied at this laboratory can be divided into two main groups: (1) a large group presenting the same general characteristics as the cases described above, and (2) single cases or small groups of cases due to a variety of agents, in some instances psittacosis-like viruses, in others unknown agents. While the occurrence of clear-cut epidemics of the former type suggests a single

etiology, it is by no means certain that the group is homogeneous with respect to its etiology. The data presented in this paper and in a previous publication indicate that a substantial proportion of the cases of this type on which neutralization tests have been done are due to this new atypical pneumonia virus. It has already been shown that the virus can cause an illness which varies in severity from a comparatively mild one to an extremely severe or, in rare instances, a fatal one. The number of cases studied is small, however, and no definite statement can be made at the present time concerning the frequency of infection with the virus in this and other areas.

The occurrence of cold agglutinins in the sera of most of those patients whose illness was shown to be due to the new virus raises the question whether a significant increase in cold agglutinin titer following a respiratory disease represents a specific response to a single etiological agent or a relatively nonspecific response to any one of a number of agents. The cold agglutination tests done in this laboratory on a large number of sera from patients in the first group have shown that more than half of the persons studied developed significant (40 or more) titers during the latter part of their illness or during convalescence. Certain other patients with clinically similar cases did not. Further combined cold agglutination tests and etiological studies must be made before it is possible to state in what proportion of pneumonias due to this virus cold agglutinin titers rise. The frequency of high cold agglutinin titers in pneumonias and upper respiratory infections due to other agents likewise remains to be determined. Even in our present state of knowledge, however, the test has proved very useful, possessing the great advantage of simplicity of performance. Its chief disadvantage lies in the fact that titers return to within normal limits in a relatively short time, thus curtailing its usefulness in epidemiological investigation.

The comparatively infrequent cases comprising the second group in which the etiology has been determined represent, for the most part, infections with viruses or rickettsiae present in a variety of species of birds, mammals, or arthropods, or occur as laboratory infections. As a group, they tend to be more severe and not in-

frequently terminate fatally. The largest number is caused by viruses in the psittacosis group, 3 of which have been isolated from human patients, namely psittacosis, the pigeon ornithosis virus, and the S-F strain (13, 17). Because of certain similarities in the clinical picture, incubation period, and pathological findings observed in psittacosis-like infections and in the prevalent type of primary atypical pneumonia, it has been suggested that these or related agents play an important part in the etiology of primary atypical pneumonia (17, 18). Evidence to support such a hypothesis is at present lacking. In this laboratory, less than 10 per cent of more than 250 cases studied have been shown to be due to viruses in the psittacosis group. We have not observed pneumonias due to the rickettsia of Q-fever or infections with the lymphocytic choriomeningitis virus in which pulmonary consolidation occurred.

Specimens of lung tissue from a considerable number of persons, in which a bacterial etiology has been definitely excluded, have failed to yield any of the viruses mentioned in this paper. This fact, together with certain observations, such as that (19) of a pneumonia of infants characterized by eosinophilic inclusion bodies, makes it seem probable that a number of non-bacterial infectious agents capable of causing pneumonia remain to be discovered.

SUMMARY

1. Clinical and laboratory data have been presented on 16 cases of primary atypical pneumonia assumed to be due to a recently isolated virus.
2. This atypical pneumonia virus is transmissible to chick embryos, cotton rats, and hamsters and is apparently not related to previously described viruses isolated from cases of atypical pneumonia.
3. The clinical picture did not differ in significant respects from that presented in other reports of epidemics of primary atypical pneumonia.
4. The pathological findings in a fatal case from which virus was isolated have been described.
5. Significant titers of cold agglutinins were demonstrated in the convalescent serum speci-

mens of all 13 patients in whom this test was performed.

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THE TREATMENT OF SUBACUTE BACTERIAL ENDOCARDITIS WITH PENICILLIN¹

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The first reports on treatment of subacute bacterial endocarditis (*S. viridans*) with penicillin were not encouraging (1). It was recognized, however, by the Committee on Chemotherapeutics and Other Agents that the subject required further study, and it was planned to treat more patients when larger amounts of penicillin, which seemed to be necessary, should be available. Meanwhile, improvement was reported (2) in some cases, and possible cure in others, when penicillin and heparin were given at the same time. It seemed desirable, however, to try out the effects of penicillin alone, given in sufficient dosage to put the matter to a critical test. The present report concerns the results in 11 cases which were managed according to the following plan.

SPECIFICATIONS FOR SELECTION OF CASES

The criteria for the selection of patients for treatment were as follows:

1. Positive blood cultures were obtained in our own laboratory.
2. The strains of non-hemolytic streptococci were all sensitive in the test-tube with a concentration of 0.1 units of penicillin per milliliter of culture medium. In all but one case, the strains actually were inhibited by smaller amounts of penicillin.
3. The patient's condition was not desperate. The known duration of the disease was not over a few months. There were no frank signs of cardiac failure. Three cases in a sense were exceptions: Case 10 in which there was incipient decompensation in association with a marked valve lesion, Case 2 who ran a stormy febrile course with over 500 colonies of *S. viridans* per ml. of blood before treatment, and Case 3 who was very ill with high fever and considerable anemia.

4. Treatment was not undertaken unless the patient agreed to stay in the hospital for 6 to 8 weeks or more for continuous therapy.

PLAN OF ATTACK

Since previous experience had shown that even large doses of penicillin over brief periods did not effect regular cures, it was decided to give the material for at least 6 to 8 weeks. It was hoped that a continuous assault of this sort might eradicate bacteria lurking in the depths of vegetations and that the latter might become organized and tend to "heal." The general plan was to give 200,000 to 300,000 units in the 24 hours by continuous intravenous drip for 3 weeks and thereafter 120,000 to 200,000 units per day in 8 intramuscular injections (5000 units per ml. of saline) for 3 to 5 weeks. Most of the patients were treated for 8 weeks without interruption. The only therapy aside from penicillin was general supportive and nursing measures and in some cases transfusions. Many of the patients were confined to bed only until the temperature fell. They were then up quietly about the ward. Frequent physical examinations were made with special reference to embolic lesions, petechiae, the cardiac signs, etc., and numerous blood cultures were taken, for the most part with penicillinase, so that the results were not confused by penicillin carried over into cultures from the blood stream. No heparin was used.

The evidences of a renal lesion were very carefully followed by means of "Addis quantitative counts" of the formed elements in the sediment to try to determine whether treatment influenced the process.

The most important clinical features are summarized in Table I and in the following brief case reports.

Case 1. A 22-year-old ditch-digger had always been mentally defective. He was also said to have had a heart murmur since childhood. He was perfectly well until 3 months before entry when he suddenly developed chills and fever with malaise and weight loss. Several blood cultures were positive for *S. viridans* and he was sent to Stanford Hospital for penicillin treatment. He was a big, heavy-set man. There was cardiac enlargement with a loud systolic murmur widely heard, moderate systolic hypertension in the upper extremities, and absence of palpable pulsations in the femoral, popliteal, and foot arteries. It was concluded that he had obstruction of the aorta and possibly an interventricular septal defect, with superimposed *S. viridans* infection. Treatment and other data are given in Figure 1 and the tables.

¹ The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for Clinical Investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

TABLE I
Summary of essential data in Cases 1 to 11

No.	Age	Sex	Heart lesion	Duration symptoms (months)	Physical findings				Renal lesions	First blood culture	Duration treatment (days)	I. V. units per day	I. M. units per day	Total units	End results	Follow-up
					Spots	Spleen	Clubbed fingers	Emboli								
1	22	M	Coarct. aorta. Septal defect?	3	+	0	0	0	+++	<i>S. viridans</i> 70 cols. per ml.	42	300,000 20 days	120,000 22 days	8,655,000	Bacteria-free and clinically well.	Follow-up period 6 months. Clinically well. Blood culture negative.
2	41	F	Mitral stenosis	3	+++	+	+	0	+++	<i>S. viridans</i> 500 cols. per ml.	65	300,000—5 da. 200,000—15 da.	120,000 45 days	9,750,000	Bacteria-free but developed cardiac failure.	Died—cardiac failure—3 mos. after completion of "P" therapy. Blood sterile. Autopsy showed healed lesion.*
3	34	M	Mitral stenosis	5	+	0	+	+	+++	<i>S. viridans</i> a few cols. per ml.	60	300,000 18 days	120,000 42 days	10,440,000	Bacteria-free. Clinically well. Renal lesion cleared.	Follow-up period 5 months. Clinically well. Blood culture negative.
4	20	M	Aortic insuff. Mitral stenosis	2 (wks.)	0	0	0	0	0	Non-hemolytic strep. 25 cols. per ml.	60	300,000 18 days	120,000 to 200,000 42 days	10,540,000	Bacteria-free and clinically well.	Follow-up period 4 months. Clinically well. Blood culture negative.
5	58	M	Mitral lesion	2	0	0	0	0	0	Non-hemolytic strep. 25 cols. per ml.	60	300,000 18 days	200,000—19 da. 120,000—32 da.	10,240,000	Bacteria-free and clinically well.	Follow-up period 4 months. Clinically well. Blood culture negative.
6	59	F	Mitral stenosis	2	+	+	+	+	+++	<i>S. viridans</i> 40 cols. per ml.	60	200,000 3 days	200,000—25 da. 120,000—32 da.	9,440,000	Bacteria-free and clinically well.	Follow-up period 3 months. No fever but tires easily.
7	64	M	Mitral lesion	2		+	0	+	+	<i>S. viridans</i> 25 cols. per ml.	60	300,000 7 days	200,000—18 da. 120,000—30 da.	9,700,000	Bacteria-free and clinically well.	Follow-up period 2 months. Clinically well. Blood culture negative.
8	18	F	Mitral lesion	5	+	0	0	0	++	<i>S. viridans</i>	60	300,000—8 da. 200,000—3 da.	300,000—15 da. 120,000—33 da.	10,000,000	Bacteria-free and clinically well.	Follow-up period 3 months. Clinically well. Blood culture negative.
9	64	F	Mitral lesion	6 (wks.)	0	0	0	0	++	<i>S. viridans</i>	60		200,000—31 da. 120,000—29 da.	9,860,000	Bacteria-free and clinically well.	Follow-up period 6 weeks.
10	29	M	Mitral lesion	1	+	+	0	+	+++	<i>S. viridans</i>	51	300,000 7 days	300,000—20 da. 200,000—24 da.	14,525,000	Bacteria-free but signs of cardiac failure.	Follow-up period 1 month. Condition said to be unchanged.
11	23	M	Aortic insuff.	3	0	+	+	7	+++	Non-hemolytic strep. 50 cols. per ml.	4	300,000 4 days		1,462,000	Sudden death on 4th day.	Autopsy.

* But see discussion of Case 2 in the text.

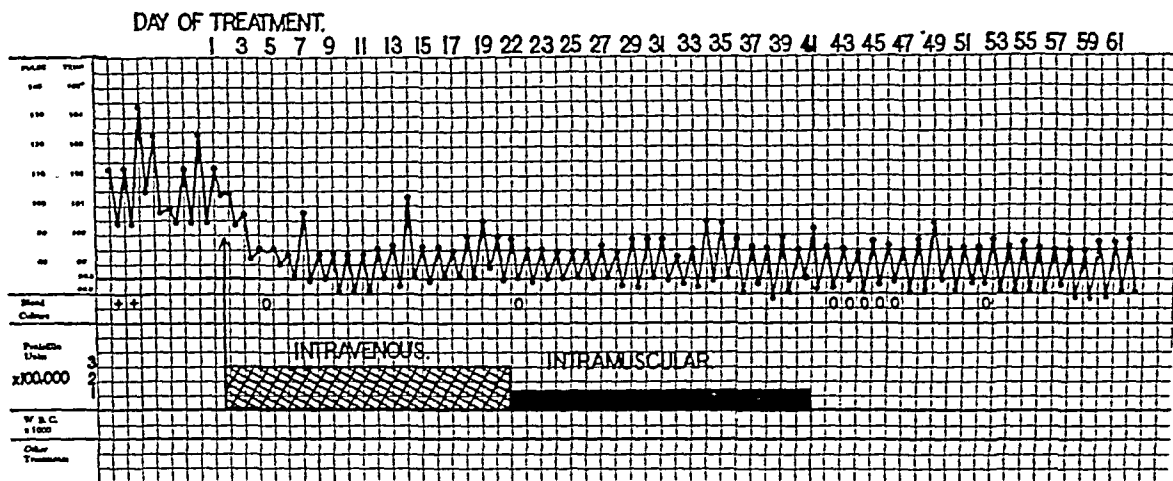


FIG. 1. COMPLETE TEMPERATURE CHART OF CASE 1 IN RELATION TO THERAPY AND BLOOD CULTURES

The persistent slight elevations of temperature after the initial drop are unexplained. Thrombophlebitis, pyrogenic reactions, reactions associated with the cardiac lesion, or small emboli are possibilities. Six months later the temperature was normal.

Temperature fell promptly but he continued to have slight elevations throughout his hospital stay. The significance of these was not clear. He left after 62 days, bacteria-free and clinically well. Last follow-up visit, 6 months after penicillin was concluded, found him well and active and bacteria-free but with physical signs unchanged. At this time, the temperature was entirely normal and his lesion was regarded as "healed."

Case 2. A 41-year-old school teacher had always been well and gave no history of rheumatic fever, but a heart murmur had been diagnosed 10 years ago. Three months before entry, she developed fever and prostration and had been ill ever since. Several blood cultures were said to be positive for *S. viridans*. On examination, she seemed very ill, with high fever and rapid pulse. She was covered with innumerable large and small petechiae. There was early clubbing of fingers. The heart was enlarged and there were typical signs of mitral stenosis. Blood cultures yielded over 400 colonies of *S. viridans* per ml. of blood. The impression of one examiner was "she is going down hill fast and a good therapeutic result would be miraculous." Penicillin was started (see Figure 2 and the tables) by continuous intravenous drip at the rate of 300,000 units per day. There was remarkably prompt improvement of general symptoms with fall in temperature although slight elevations continued. There was rapid clearing of the petechiae although some new ones appeared for 2 weeks. The blood stream was cleared in a few days and all subsequent cultures were negative. After being rendered bacteria-free, she developed curious red tender symmetrical swellings of all fingers and toes, apparently associated with acute progression of "clubbing." Studies of blood vessels and of capillary circulation were not remarkable. Later she developed a frank arthritis of the fingers with smooth shiny skin. Signs of cardiac failure, not present on

entry, now began to develop and she died on the 164th day of hospital stay in an acute paroxysm of dyspnea after being bacteria-free for over 5 months. The temperature at this time had been entirely normal or sub-normal for several weeks.

Autopsy. Pertinent points in the autopsy report are as follows (Dr. J. B. McNaught): "The mitral valve as viewed from above prior to opening is moderately irregular, nodular, and apparently not efficient, but not decidedly stenosed. Upon opening it, the circumference is 9 cm., but both flaps in the midportion have thick nodular, rather rigid shrunken edges. The anterior cusp is considerably shortened in the distance from base to free edge and has a 0.4 cm. round hole with raised edges 0.5 cm. from the free edge. There are no soft vegetations or thrombi and calcium can be palpated in some of the nodules. Two chordae tendinae are thickened up to 0.5 cm. and shortened and the endocardium beneath the anterior flap is thick and white. There is a 1.5 x 1 cm. granular patch on the endocardium of the left ventricle 1.5 cm. below the aortic valve. The ventricular surface of the posterior flap is roughened with calcification."

Microscopic examination of the heart showed: "The mitral valve leaflets are greatly thickened centrally by hyalinized fibrous tissue with very few nuclei, and there are small areas of calcification deep within the fibrous tissue. There are also surface areas of more cellular well vascularized fibrous tissue with a few lymphocytes, and polymorphonuclear leukocytes and scattered large mononuclear cells some of which contain hemosiderin. These areas have the appearance of organization of thrombotic tissue and occasionally enclose small areas of necrotic thrombus some of which contain many Gram + cocci singly, by pairs and in short chains. The outermost surface is replaced by areas of pink staining

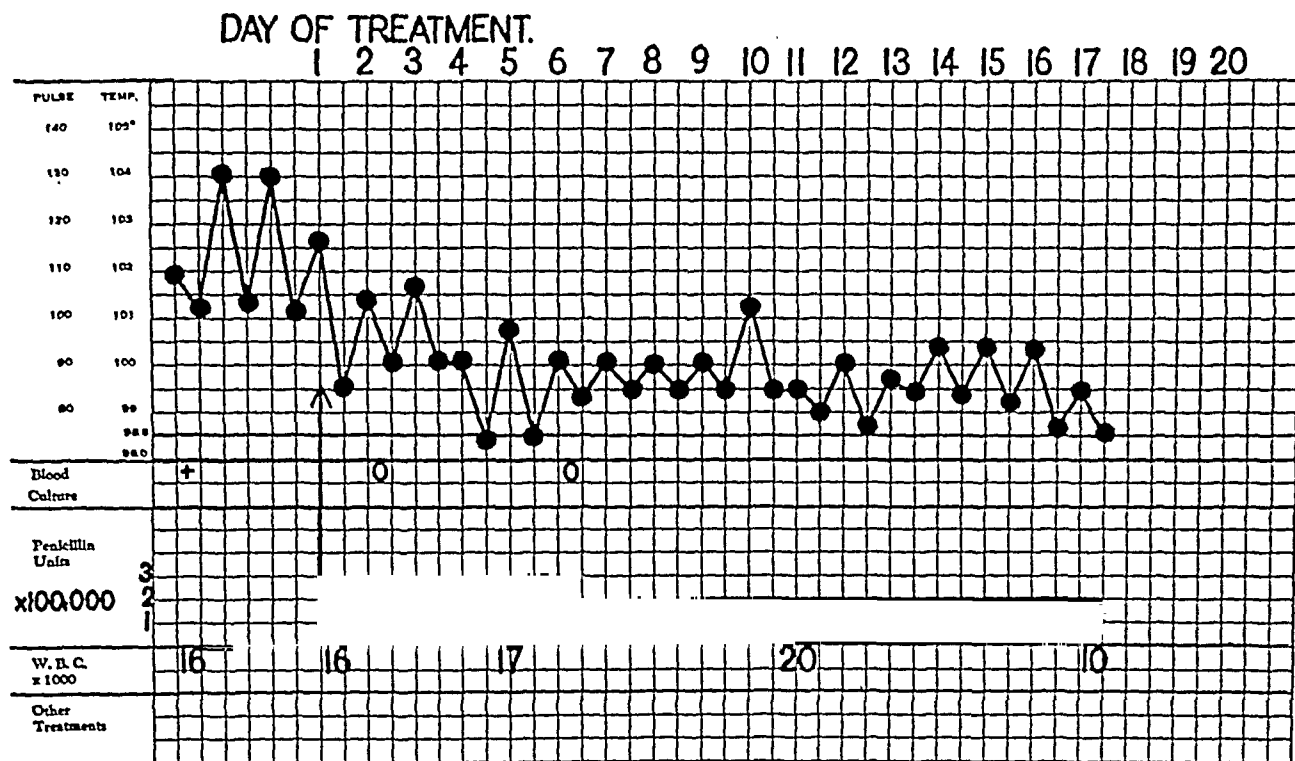


FIG. 2. SHOWS TEMPERATURE CURVE FOR FIRST THREE WEEKS OF TREATMENT

She continued to have a variable low fever throughout her long hospital stay (even after 3 blood cultures were sterile) until the last few weeks when the temperature became subnormal. As in Case 1, the exact cause of these elevations was not always clear.

practically acellular thrombotic material in which fibroblasts are growing from beneath. They sometimes contain a few polymorphonuclear leukocytes. Many of these nodules are quite cellular and vascular as though recently organized, and an occasional such nodule contains within its depths small islands of necrotic thrombus rich in pleomorphic Gram positive cocci singly and in short chains. None of these bacteria-laden areas are found exposed on the surface of the valve.

"The chordae tendinae are thickened, similar to the valve. The endocardium of the auricle and ventricle are roughened by organizing vegetations in which no bacteria are found. The myocardium at the base of the mitral valve and the apex of the papillary muscles is irregularly scarred. Elsewhere, there is a slight increase in fibrous tissue about many of the blood vessels but no thickening of their intima and there are no cellular collections suggesting Ashoff bodies. The myocardial fibers are normal with no abnormal cellular infiltrations. Large branches of the coronary arteries show mild subintimal fibrosis.

"As regards the kidneys, in general the histology in both is normal except for congestion. However, meticulous search reveals rare glomeruli which are partially adherent to their capsule, slight capsular proliferation, and slight tuft fibrosis which may well represent a late stage of focal glomerulonephritis with no fresh hyalin lesions. No blood is found in capsular spaces, but rare tubules contain red blood cells. There is granular mate-

rial in some of the tubules and rare hemoglobin stained casts. The right kidney contains a sunken area of old infarction with diffuse fibrosis, fibrosed glomeruli, areas of necrosis containing hemosiderin and shadows of necrotic tubules. There are moderate numbers of lymphocytes in this area which invade the adjacent more normal renal tissue and here a few tubules contain polymorphonuclear leukocytes. A small artery at the apex of the infarct is obliterated by fibrous tissue. The left kidney contains a similar but narrower and more fibrosed area."

These findings raise a number of problems of interest and importance. At the time of autopsy, the lesions appeared to be healed; no coarse vegetations were any longer present, only small partly calcified flat plaques remained. Culture from the heart's blood yielded no growth and no bacteria were seen in smears from the surface of the lesions. Many blood cultures had been negative over a period of several months and the temperature was normal for a month before death. None the less unmistakable gram-positive cocci were seen in the depths of the "healed" valve lesions. A good deal of pleomorphism and variability in staining reaction were observed. Were these bacteria dead and sealed off in pockets of old organized vegetation? Were they viable, but sealed off, or were there actually streptococci in the blood stream which were not recovered by the relatively crude method of blood culture? Had she not died of cardiac failure, would there later have been a clinical relapse of bacterial endocarditis? None of these

questions can be answered definitely and one can only record the facts and wait for longer follow-up periods in other cases. The findings do however support the position that penicillin treatment should be given over a long period of time, perhaps over many months, continuously or intermittently.

Case 3. A 34-year-old accountant had had polyarthrititis at the age of 15. A heart murmur was known to be present since that time. Five months before entry, he developed an indefinite febrile illness. There was no benefit from 4 months' treatment with sulfonamides. On examination, he appeared febrile and ill and there was marked weight loss. There were typical signs of mitral stenosis, a palpable spleen, early clubbing of fingers, and a few petechiae. He was moderately anemic and there was evidence in the urinary sediment of a mild glomerulitis. The blood culture yielded non-hemolytic streptococci. He was given 300,000 units of penicillin daily by intravenous drip for 18 days and then 15,000 units intramuscularly every 3 hours for 32 days—a total of 10,440,000 units in 2 months. The high temperature on entry fell in a few days and remained essentially normal with the exception of 2 pyrogenic reactions (see Figure 3 and the tables). He gained 20 lbs., recovered his appetite and good spirits, and changed from what appeared to be a dying man to one who seemed perfectly well. The heart murmur persisted unchanged and the pulse remained slightly rapid. The signs of the renal lesion disappeared. Blood cultures promptly became sterile and remained so on many occasions.

Follow-up studies after a period of 5 months showed him to be clinically well with entirely normal temperature and negative blood cultures. He had been actively at work with no bad results.

Case 4. A 20-year-old truck driver had always been well except for rheumatic fever at the age of 5 followed by signs of aortic insufficiency. Two weeks before entry he felt tired and developed high irregular fever. On examination, he was a big healthy looking young man with typical signs of aortic insufficiency. The blood pressure was 160/?. The spleen was not palpable; there were no petechiae or embolic phenomena. Blood culture yielded 25 colonies per ml. of non-hemolytic streptococcus. He received a total of 10,540,000 units of penicillin over a period of 60 days (see Figure 4 and tables). In this case, although the blood stream was promptly and permanently sterilized the temperature fell quite gradually. It was, however, perfectly normal for some days before discharge. He gained weight, all symptoms disappeared, and he seemed entirely well. He was carefully examined after a follow-up period of 4 months and still appeared "cured" with normal temperature and negative blood cultures. The signs of aortic insufficiency were unchanged.

The persistence of fever in this case, whatever the cause may have been, is an argument in favor of prolonged penicillin therapy.

Case 5. A 58-year-old painter had a bout of hemoptysis and dyspnea 4 years ago and was then told he had a leaky heart valve although there was no history of

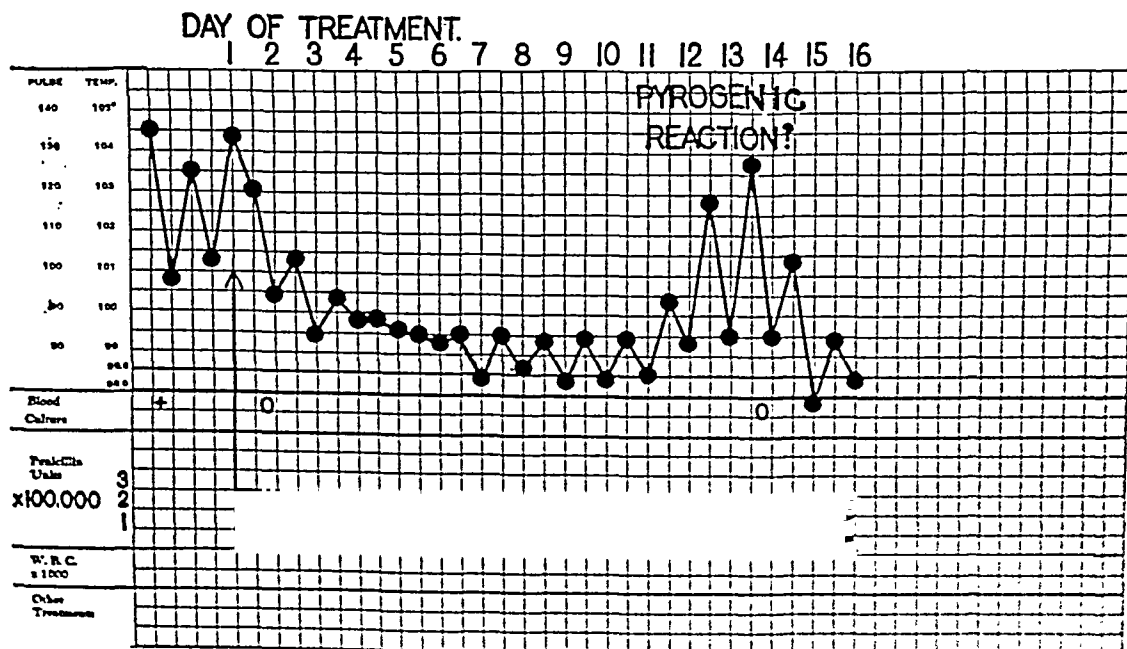
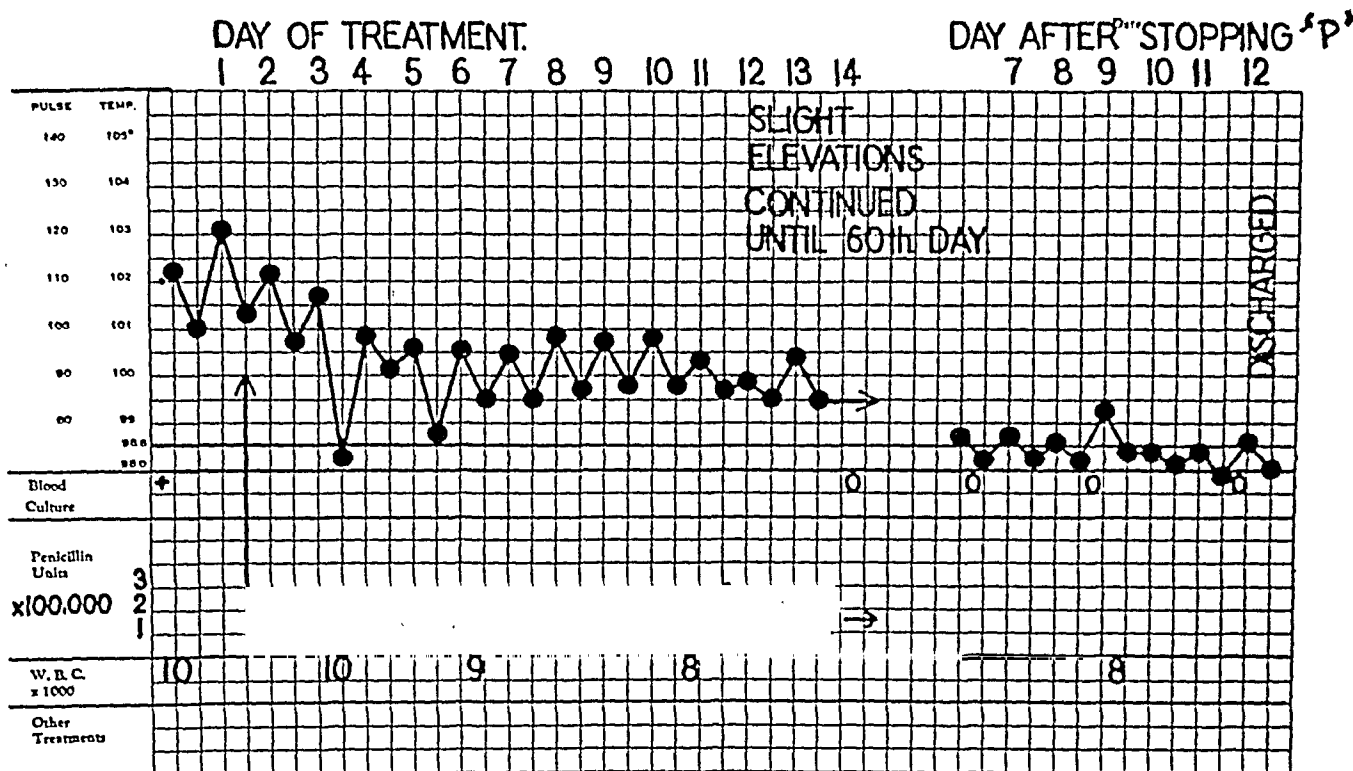


FIG. 3. TEMPERATURE CURVE FOR FIRST 16 DAYS OF TREATMENT

Remarkably prompt fall. With the exception of another febrile reaction from the 17th to the 19th days, probably pyrogenic, the curve remained essentially normal until discharge on the 83d hospital day.



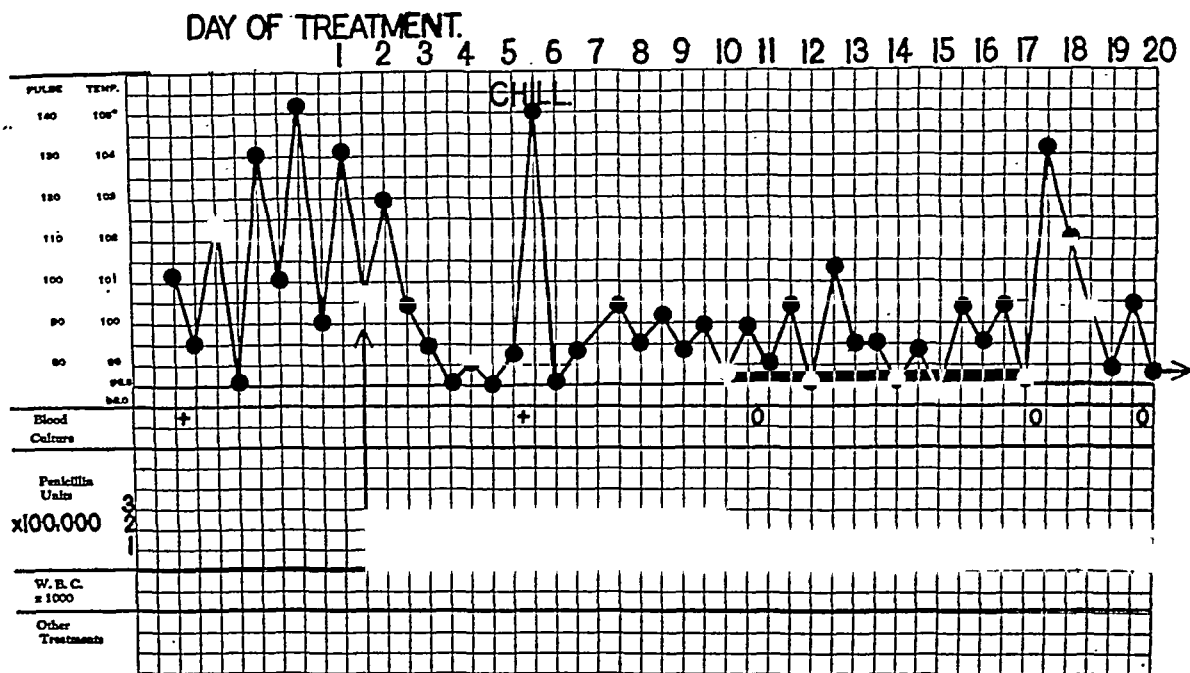


FIG. 5. TEMPERATURE CURVE FROM CASE 5 SHOWING FIRST 3 WEEKS OF TREATMENT

Occasional spikes of fever occurred during the first months. Thereafter the temperature was normal.

loss. On examination, he was a little pale and looked tired and worn. The first sound at the apex was followed by a loud rough murmur, typical of organic valvular disease. The spleen was readily felt. There was a

tender area over the tendons of the right wrist. Two blood cultures each yielded about 25 colonies of *S. viridans* per ml. He was treated for 60 days with penicillin, receiving a total of 9,700,000 units. Blood culture

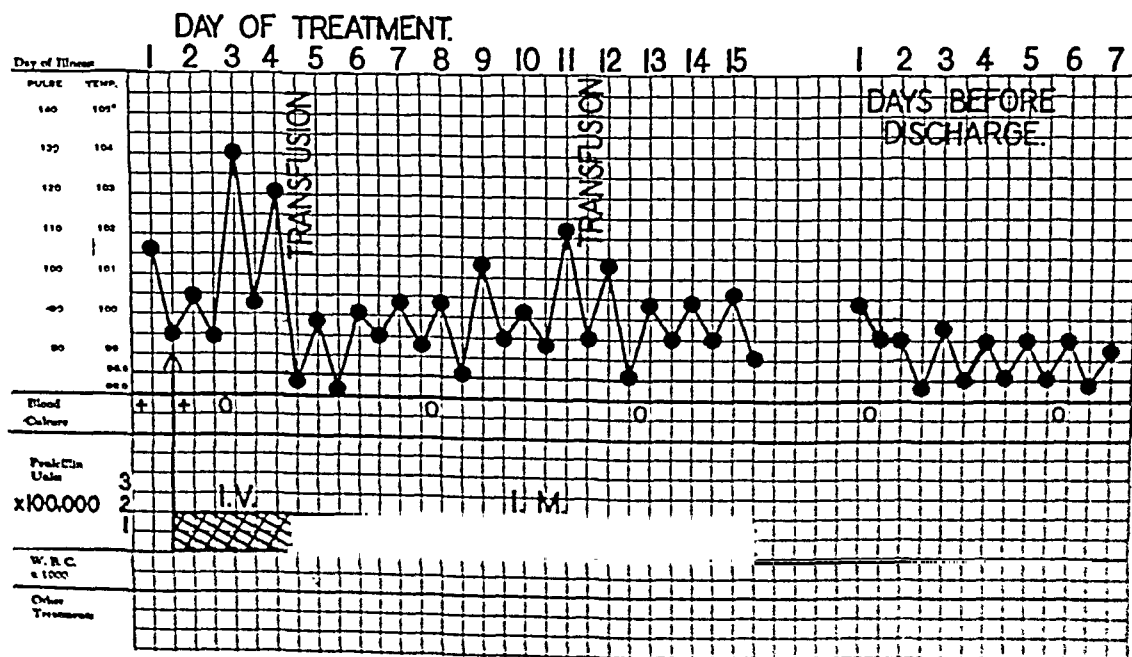


FIG. 6. TEMPERATURE CHART OF CASE 6 FOR FIRST 2 WEEKS OF TREATMENT AND OF LAST WEEK IN HOSPITAL AFTER PENICILLIN WAS STOPPED

(with penicillinase), made 2 hours after the intravenous drip of penicillin was started, yielded no growth and all subsequent cultures were sterile. There was a prompt drop of temperature (see Figure 7 and the tables) and within a few days he felt much better and ran an essentially uneventful course. On discharge on the 64th day, and 4 days after stopping penicillin, the temperature was normal and he felt perfectly well. The spleen was no longer palpable, but the heart sounds were unchanged. Follow-up visit 2 months later found him well and at work, and blood culture was sterile.

Case 8. An 18-year-old housewife had had rheumatic fever at the age of 10. She was in bed for a long time, was left with a heart murmur, but was essentially well. About 4 months before entry, she developed fever, malaise, joint pains, and palpitation, and 7 blood cultures were reported positive for *S. viridans*. Sulfadiazine was given without benefit in another hospital. On examination, she did not appear very ill. There was moderate fever. There was a thudding first sound at the apex followed by a loud musical systolic murmur. A soft echoing diastolic murmur was also heard to left of sternum. It was thought that she probably had an old rheumatic mitral lesion with subacute bacterial endo-

carditis, and blood culture again yielded *S. viridans*. There were a few petechial spots but the spleen was not felt. There was no evidence of a renal lesion. She received 300,000 units of penicillin daily by intravenous drip for 8 days and slightly smaller amounts for the next 3 days. Thereafter intramuscular injections were given in amounts of 120,000 to 200,000 units daily up to 60 days—a total of 10,000,00 units. There was a prompt drop of temperature to normal (see Figure 8 and the tables). The blood cultures became negative and she felt well. Her course was uneventful except for 2 spikes of fever, probably due to pyrogenic solutions, and she left the hospital well, but with the heart signs unchanged. Follow-up studies 6 weeks later showed her to be the picture of health, with no symptoms, normal temperature, and negative blood culture.

Case 9. A 64-year-old woman had had "rheumatic fever" 18 years ago. Six weeks before entry, she developed chills and fever, and blood cultures were found positive for *S. viridans*. She was treated for 4 weeks with sulfathiazole without benefit. Examination showed a thin, tired, febrile elderly woman. The heart was enlarged, the rate 140-150. There were typical signs of mitral stenosis. The spleen was not felt; there were no

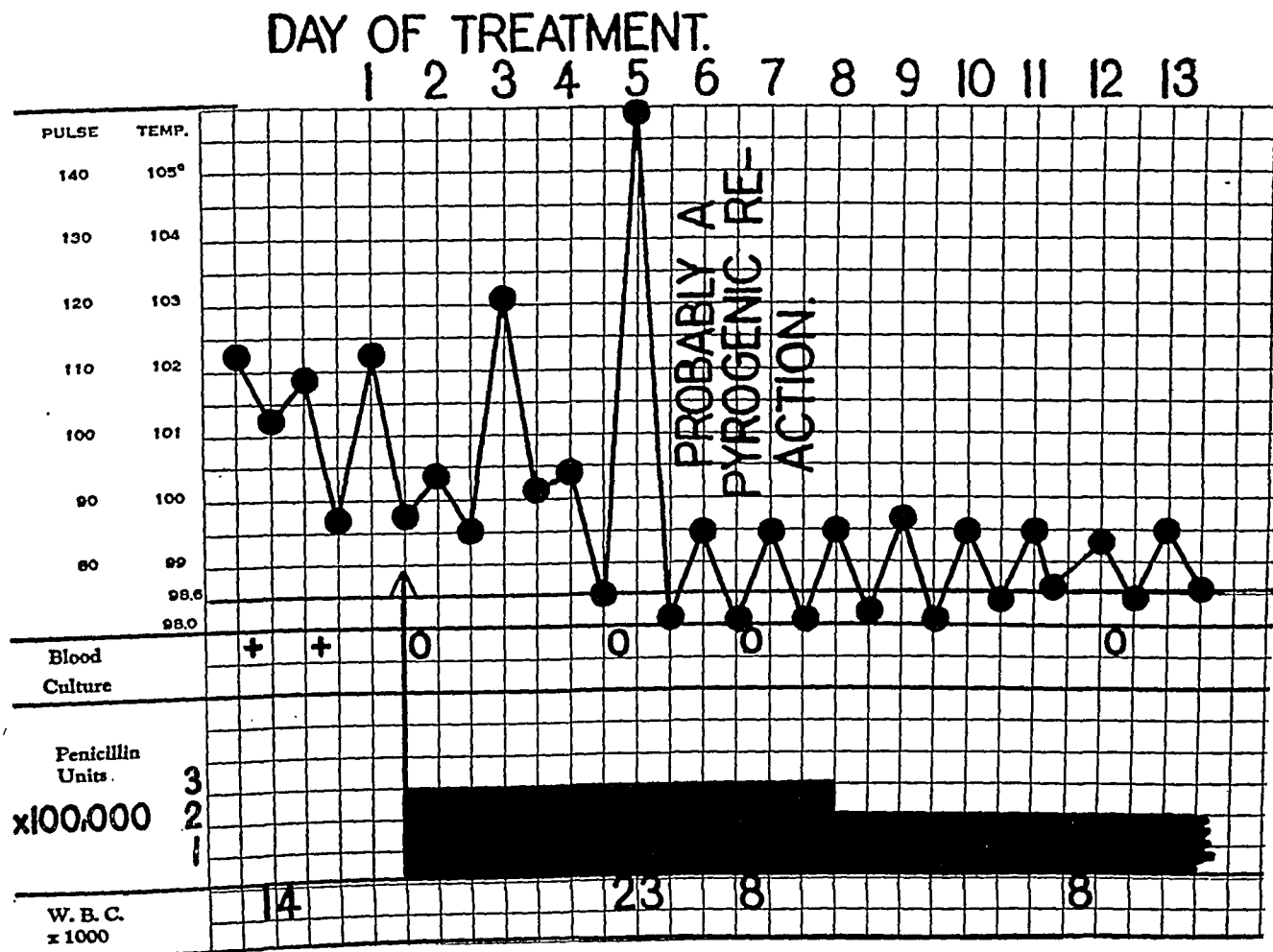


FIG. 7. TEMPERATURE CURVE OF CASE 7 FOR FIRST 2 WEEKS OF TREATMENT
From this time on, aside from occasional elevations, the temperature was essentially normal.

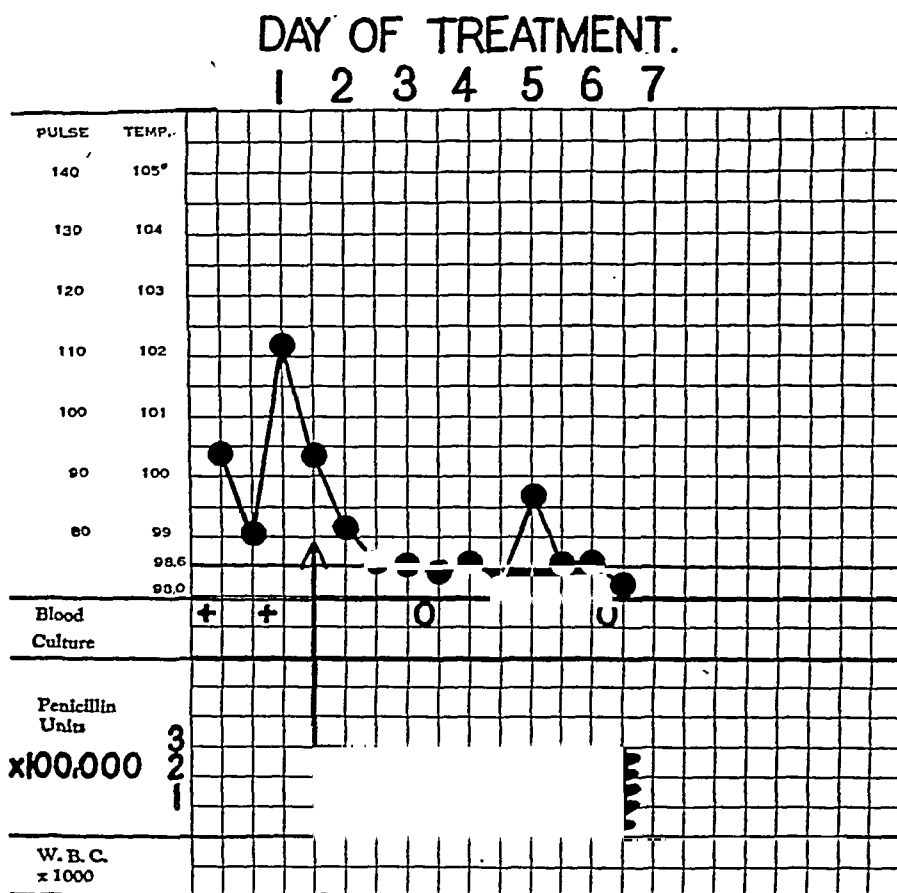


FIG. 8. TEMPERATURE CURVE OF CASE 8 FOR THE FIRST WEEK OF TREATMENT

Except for febrile reactions on the 9th and 32d days, probably from pyrogenic solutions, the temperature remained essentially normal.

petechiae. Blood culture yielded 70 colonies of *S. viridans* per ml. This patient received intramuscular injections of calcium penicillin from the start, 200,000 units daily for the first month and 120,000 units daily for the second month—a total of 9,860,000 units. There was a gradual fall in temperature but slight elevations persisted up to discharge (see Figure 9 and the tables). Blood cultures promptly became sterile and all symptoms cleared up. She left the hospital clinically well. Six weeks later she was in the hospital for a week for follow-up studies. The highest daily rectal temperatures were about 37.5° C., blood culture was sterile, and she felt well although the heart signs were unchanged. Investigation of kidneys by excretory pyelography, because of persistent changes in urinary sediment, revealed an appearance suggestive of polycystic kidneys.

Case 10. A 29-year-old bookkeeper had no history of rheumatic fever but was said to have had an enlarged heart since childhood. He had been short of breath on effort for a long time. About a month before entry, he had what was called "flu," followed by bilateral flank pain for 12 days. Blood cultures were positive for *S. viridans*. Examination showed an acutely ill man with

high fever, greatly enlarged heart, systolic and presystolic apical murmur, palpable spleen, and tenderness in right flank. There was no frank decompensation at this time. He was started on penicillin intravenously (see Figure 10 and the tables) but seemed unable to accommodate the required amount of fluid. There were several episodes which seemed to be associated with pulmonary and renal infarcts. It was thought that some of the spikes of high fever might be associated with transfusion reactions but even after these were stopped the temperature stayed high until the 21st day of treatment when it fell rapidly to almost normal. The blood cultures, however, were negative on the second day of therapy and remained so throughout. He was given penicillin intensively for 60 days mostly by the intramuscular route (Figure 10). As his infection improved, frank signs of cardiac failure developed and he was sent to another hospital for continued rest.

Here, then, is an instance of probable extirpation of the infection but with sufficient damage to precipitate cardiac failure. It is also of note that this patient's strain was not inhibited in the test tube with concentrations of penicillin less than 0.1 units per ml. of media.

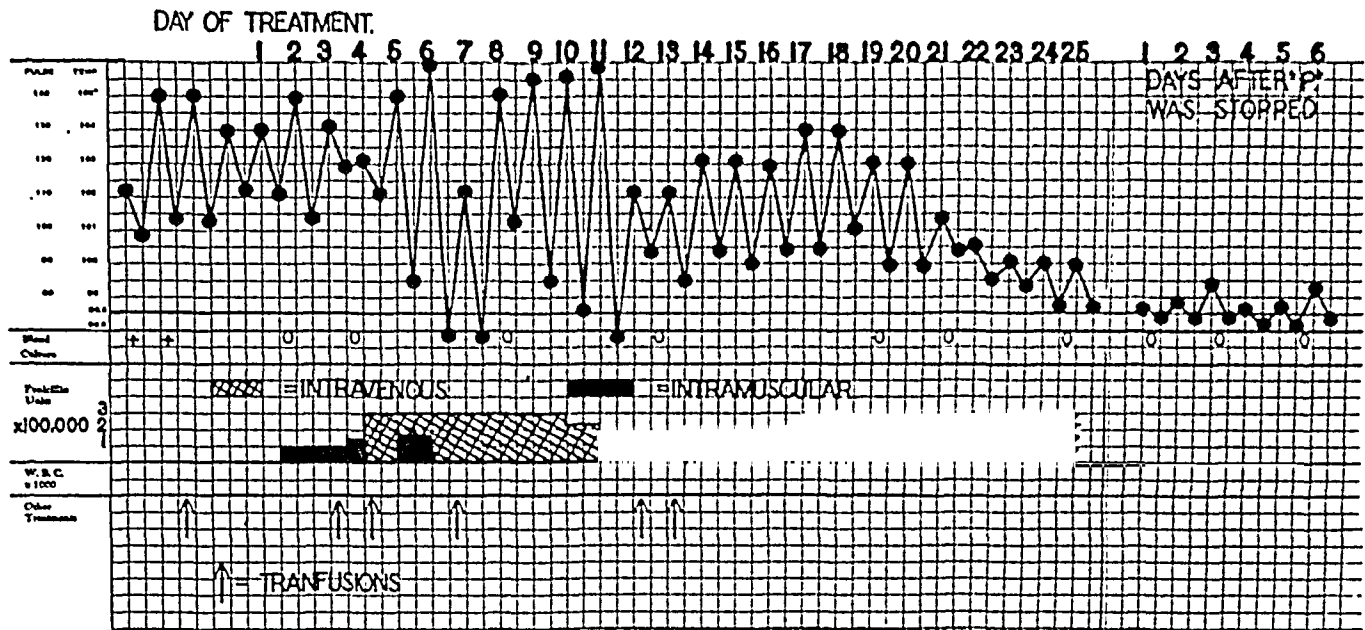


FIG. 9. TEMPERATURE CURVE FROM CASE 9

In spite of this relative resistance, blood cultures promptly became negative.

Finally, the importance of prolonged treatment is brought out, as one might well have considered him a failure at the end of 3 weeks when the fever was still high.

Case 11. A 23-year-old man had had "St. Vitus dance" at the age of 12 followed by "heart trouble." He had gotten along all right, however, until 3 months before entry when he developed increasing fatigue, cough, swollen ankles, and fever. A blood culture was positive for *S. viridans* and he was sent to Stanford Hospital

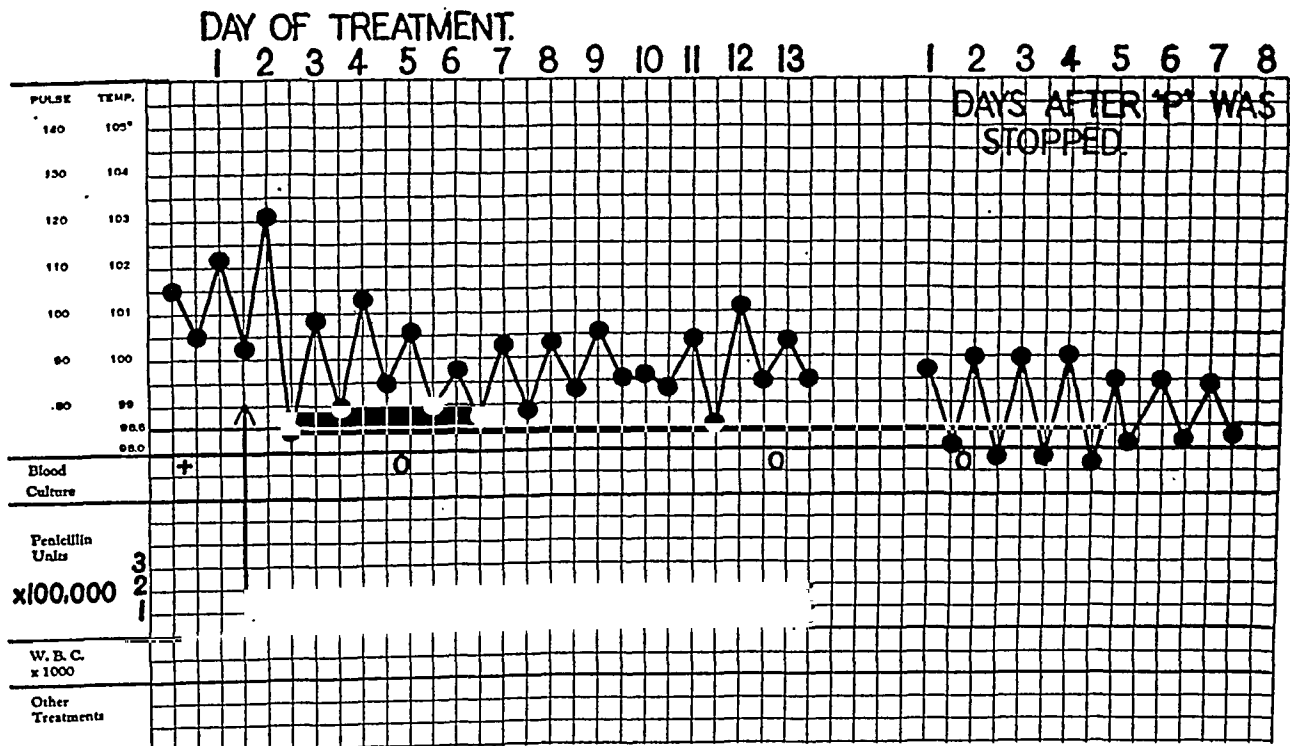


FIG. 10. TEMPERATURE CURVE FROM CASE 10 SHOWING FIRST 25 DAYS OF THERAPY AND FIRST WEEK AFTER PENICILLIN WAS STOPPED

It was only after 3 weeks of treatment that fever subsided.

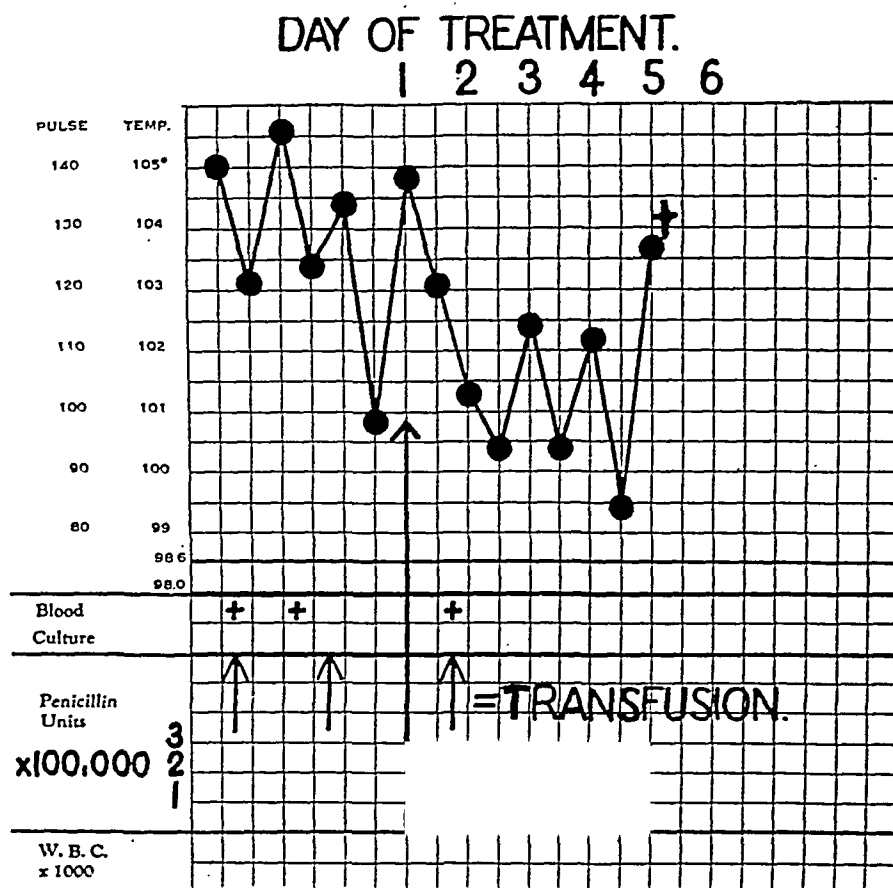


FIG. 11. TEMPERATURE CURVE FROM CASE 11

for penicillin therapy. On examination, he looked sallow and tired. There was clubbing of the fingers but no petechiae were seen. The spleen was palpable. He was somewhat dyspneic. The heart was markedly enlarged and there was a loud aortic diastolic murmur. The blood pressure was read at 175/?. There were some râles at the bases. There was high fever and marked anemia with packed cell volume of only 32, red blood cells 2.9 M, and white blood cells 18,000. The urine showed the sediment of a marked glomerulitis. Blood culture on entry yielded 50 colonies of non-hemolytic streptococci per ml. of blood. The strain was highly sensitive to penicillin and although he did not quite meet the specifications which we had laid down, it was decided to treat him. Penicillin was started by intramuscular injection every 3 hours at the rate of 300,000 units in the 24 hours. The temperature fell somewhat with prompt improvement in well-being. On the fourth day, out of a clear sky, there was sudden collapse with rapid feeble pulse, prostration, sweating, and death in a few hours (see Figure 11 and the tables).

Autopsy showed a huge oval vegetation loosely attached to the aortic valve but there was no coronary artery or pulmonary embolus and no rupture of a my-

cotic aneurism. In short, no cause of death was found. It was thought that the mobile vegetation might have occluded the mouth of a coronary artery but this was purely speculative. At any rate, it seemed clear that penicillin had nothing to do with his death. The same lot was given to many other patients without reaction. There were no intravenous injections.

While the case is included in the series, it really can not be considered a "penicillin failure."

RESULTS

Immediate effects

The first and one of the most striking effects of penicillin therapy in many cases was an improvement in general well-being. Within a few days, in some cases within 24 hours, the patients insisted they felt better. One got the impression that "toxemia" had been lessened; appetite improved, and there was a desire for increased activity, reading, etc. To some extent, these changes went hand in hand with drop in temperature, but by no means altogether.

Effects of treatment on fever

In 8 of the 11 cases, there was a prompt and striking drop of temperature to a practically normal level of not over 37.5° to 38° C. (rectal). One patient died on the 4th day of treatment (Case 11), and in Case 10, the temperature showed no definite decline for the first 3 weeks of treatment but then came down. This patient had a strain of streptococcus which was relatively resistant in the test tube. After the initial drop of fever, the temperature remained essentially normal in some cases. In others, a flat curve was disturbed from time to time by greater or lesser elevations, perhaps due to emboli, or perhaps to pyrogenic reactions or to phlebitis associated with intravenous therapy. In still other patients, after the initial drop, a low fever continued more or less throughout the hospital stay. This we were inclined to regard as part of the disease perhaps associated with absorption and with healing of lesions even after the patient was "bacteria-free." However, an examination of the curves obtained during the last week of hospital stay, after penicillin injections had been stopped, showed the highest temperature (rectal) in any case to be under 38° C.

All in all the fever curves suggest that treatment should be continued over a long period in order to obtain the most secure results, even though an occasional patient may be "cured" with a short course of penicillin.

The findings described are illustrated in Figures 1 to 11.

The effect of penicillin on the blood culture

When the patient is receiving large doses of penicillin, enough of the antibiotic may be carried over from the blood stream to inhibit organisms which otherwise could be grown in culture. For this reason, "penicillinase" (3) was added to the media with many of our blood cultures in order to prevent spurious "negative" results. As seen in Table II, the blood stream was sterilized with great promptness in practically all cases. Indeed, after the first few days, no positive culture was ever obtained in any patient, although in the whole series, 52 cultures were made during the period of penicillin therapy and 71 cultures were made during the follow-up period after peni-

cillin was stopped. This differs somewhat from the experience of others (4) with patients treated for relatively short periods, to the effect that there is a marked tendency to relapse within a few days to a few weeks after the discontinuation of penicillin. We are inclined to ascribe the results obtained so far in this series to the *time factor*—the continuous treatment over a long period (6 to 8 weeks).

The detailed data are given in Table II. It is to be noted that there was no relation between the degree of bacteremia and the readiness with which the blood stream was sterilized, or indeed with the subsequent course. The paramount point seems to be the sensitiveness of the strain in question, although further statistics on this point are necessary.

Case 5 is of particular importance since a spike of fever on the fifth day was associated with a positive blood culture (see Figure 5). This suggests that viable organisms may lurk in the depths of the vegetations for some time.

Petechial spots

The relation of petechial spots to bacteremia has not, as far as we know, been exactly established. It seems of particular interest, therefore, to study the occurrence of petechiae before and after penicillin therapy. Among the 11 patients, there were 4 in whom none was seen at any time. Case 2, on entry, had the most profuse eruption we have ever observed in bacterial endocarditis. The skin, conjunctivae, and buccal mucosa were peppered with innumerable spots. Showers of fresh lesions continued for the first 4 days of penicillin therapy; thereafter, fewer spots appeared; and after the 2 weeks of therapy, no more were seen. The blood culture on the other hand was negative after one day of treatment and bacteria were never again grown from the blood stream.

The following table (Table III) summarizes the findings in all the cases. It is seen that petechiae continued to appear in many cases long after bacteria could no longer be obtained from the blood. Negative blood cultures do not, of course, prove the complete absence of bacteria from the blood stream, but petechiae ultimately were no longer found and this we were inclined to associate with "healing" of the lesions.

TABLE II
Effect of penicillin therapy on the blood culture

Case no.	Blood cultures before "P" was started	First culture after "P" was started	Subsequent cultures (number)			
			During "P" therapy		After "P" therapy was concluded	
			Neg.	Pos.	Neg.	Pos.
1	<i>S. viridans</i> —70 cols. per ml. <i>S. viridans</i> —25 cols. per ml.	Neg. (4th day)	4	0	10	0
2	<i>S. viridans</i> —400+ cols. per ml.	Neg. (after 1st day)	2	0	12	0
3	<i>S. viridans</i> —a few cols. per ml.	Neg. (4th day)	4	0	9	0
4	Non-hemolytic strep.—25 cols. per ml.	Neg. (1 month)	1	0	10	0
5	Non-hemolytic strep.—25 cols. per ml.	Pos. (5 day) a few cols. per ml. associated with chill and fever	6	0	10	0
6	Non-hemolytic strep.—40 cols. per ml. Non-hemolytic strep.—25 cols. per ml.	Pos. (1 day) less than 1 col. per ml. Neg. (2 day)	10	0	4	0
7	<i>S. viridans</i> —26 cols. per ml.	Neg. (2 hours)	10	0	3	0
8	<i>S. viridans</i> —2 cols. per ml. <i>S. viridans</i> —8 cols. per ml.	Neg. (3 day)	3	0	9	0
9	<i>S. viridans</i> —70 cols. per ml.	Neg. (5 day)	7	0	2	0
10	<i>S. viridans</i> —8 cols. per ml. <i>S. viridans</i> —2 cols. per ml.	Neg. (2 day)	6	0	2	0
11	Non-hemolytic strep.—50 cols. per ml. Non-hemolytic strep.—50 cols. per ml.	Pos. (1 day) 15 cols.	No more taken—died on 4th day of treatment.			

Embolic phenomena

Gross embolic phenomena were not always easy to identify since bouts of fever might be due to pyrogenic reactions from penicillin solutions or to phlebitis set up by intravenous injections. However, in 5 cases, there was no suggestion of emboli at any time. In Case 7, occasional elevations

of temperature which may have been associated with emboli persisted up to the 50th day of treatment. Case 6 had splinter hemorrhages and painful finger tips up to the 45th day. In Case 3, definite splenic and pulmonary infarcts occurred until the 33d day. Case 10 had frequent emboli to lungs, kidneys, and other situations up to the 28th day. In Case 5, spikes of fever—doubtless associated with emboli since there was a positive blood culture in one such episode—persisted through the 29th day, and in Case 2, there were probable embolic episodes for the first 3 weeks.

It seems clear, then, that emboli may continue long after blood cultures are negative. This one might expect because even if the lesions have been sterilized, particles of vegetation would probably continue to break away during the early stages of healing.

Palpable spleen

In 6 of the 11 patients, the spleen was not felt at any time. In Case 6, the edge was felt up to

TABLE III
Relation of petechiae to positive blood culture

Case no.	Blood culture negative (day of treatment)	Petechiae
8	3 (first culture)	Occasional up to 35th day of treatment
3	4 (first culture)	Occasional up to 33d day of treatment
6	2 (second culture)	Occasional up to 29th day of treatment
10	2 (first culture)	Occasional up to 28th day of treatment
2	2 (first culture)	Profuse for 4 days; some up to 14th day of treatment
5	11 (second culture) First culture positive on 5th day	Very few petechiae up to 7th day of treatment
1	4 (first culture)	None seen after treatment was started

the 31st day but not thereafter. In Case 10, the spleen, at first 3 cm. below the costal margin, could not be felt after the 21st day, and in Case 3, the spleen which had been readily felt was no longer palpable after the third week of therapy. In Case 7, the spleen which was noted several finger-breadths below the costal margin on a number of occasions was never felt after the fifth day of therapy. In another patient, not included in the present series and still under treatment, the spleen which at first was a full hand's breadth below the costal margin receded almost completely within the first week of therapy. The exact anatomical explanation of decrease in size of spleen is not clear, but is probably associated with diminution of infiltration of certain cell types (5).

Clubbing of fingers

In the present series, perhaps because many of the cases were early, clubbing of the fingers was seen in only 4, or 36 per cent. In Case 2, clubbing already present on entry progressed acutely with symmetrical redness, swelling, and tenderness of the ends of all fingers and toes. Case 11 died on the fourth day of treatment so the course of his clubbing under penicillin could not be observed; in Case 6, the appearance present on entry seemed to become more marked as the patient improved, but in Case 3, slight clubbing definitely receded under treatment. The observations are too limited to justify further comment.

Change in cardiac physical signs

Since most instances of subacute bacterial endocarditis occur in connection with previously damaged valves, the question of how much these structures are further injured by the infection—especially with reference to impairment of cardiac function—becomes of great importance. In 7 of the 11 cases, no change in the character of murmurs or other physical signs was noted during the entire period of observation. Case 3 developed a moderate tachycardia (rate approximately 100 per minute) which had not been present before treatment. No definite explanation such as hyperthyroidism could be proved. In Case 6, there was a change in the quality of the first sound at the apex which became "less snapping" and the systolic murmur became much louder and almost

"musical." Case 2 was of particular importance since there was an autopsy. Five months after entry and 3 months after penicillin treatment was concluded, it was noted that "The cardiac signs are now different from those on entry. The first sound (at apex) is less snapping and is followed by a loud systolic murmur well heard in the back (this loud murmur not present at first). There has doubtless been some anatomic alteration of the valve." At autopsy there was mitral stenosis but what had obviously been larger vegetations were now represented by very small flat hard and to some extent calcified patches. The net result however was marked destruction of the valve, which perhaps promoted the cardiac failure from which the patient died 3 months after treatment was completed.

These observations suggest that, unless treatment is started early in the disease, irreversible damage to valves may occur, even though penicillin may extirpate the infection.

Changes in cardiac function

The points discussed in the last paragraph open the question of possible changes in cardiac function in connection with bacterial endocarditis. According to some writers, the frequency of cardiac failure in this disease, as well as the presence of myocardial changes, has not been sufficiently emphasized (6, 7). Other pathologists have not been impressed by the importance of such lesions in bacterial endocarditis. It seemed of particular interest therefore to look for signs of cardiac failure, if any, in patients rendered bacteria-free by penicillin. Of the 11 patients, only 1 (Case 11) showed evidence of cardiac failure before treatment. There were dyspnea, edema, râles at the bases, engorgement of the liver, tachycardia, and gallop rhythm. This patient died on the fourth day of penicillin therapy, possibly from obstruction of a coronary artery by a mass of vegetation. In 6 cases, there was no suggestion of cardiac failure at any time during our period of observation. Case 3 developed a moderate tachycardia which persisted during the follow-up period, but he was very active and without symptoms. In Case 10, there was no frank failure on entry but gradual development of mild decompensation with dyspnea, basal râles, swollen liver, edema, and weakness which has necessitated continuous hos-

pitalization although he is afebrile and bacteria-free. Case 2 (q. v.) gradually developed failure after penicillin treatment was completed. There was dyspnea, edema, râles, and increased venous pressure. These symptoms were intractable under standard cardiac therapy and she died in the hospital. Case 6, finally, at the end of the follow-up period felt very tired and became short of breath on slight effort.

In summary, then, in 3 of 11 patients, in spite of prompt clearing of the active bacterial infection, evidence of cardiac weakness supervened. These findings suggest that the insult from such infection may so damage valves or myocardium as to cause an irreversible impairment of cardiac function. This hazard must be taken into account in evaluating the ultimate outlook for patients with bacterial endocarditis "cured" by penicillin.

Changes in electrocardiogram

Electrocardiograms were taken from time to time in all the patients. In 8 cases, there were no significant changes. In Case 6, minor T wave and axis changes occurred, in Case 2 there were quite marked changes in T waves and in electrical axis, and in Case 10 there were changes in P waves, T waves, and axis. It is of interest that all these 3 patients developed more or less evidence of cardiac failure (see preceding paragraph).

Blood count

Only 2 of the patients were very anemic before penicillin therapy was started. The count on entry and the last count before discharge are shown in Table IV. It is seen that the blood was essentially normal in all cases at the end of treatment although transfusions were given to 6 patients. Leukocyte counts moderately elevated before therapy were all normal before discharge.

Renal lesions

Careful studies of the urinary sediment by the methods of Addis were done (C. D. A.) in order to study the effect of penicillin therapy on the renal lesions of bacterial endocarditis. It is not the purpose now to go in detail into the exact nature of these lesions but it is generally agreed that some sort of glomerulitis is the outstanding finding, and that the presence of abnormal num-

TABLE IV
Blood counts on entry and on discharge

Case no.	On entry			Before discharge			Transfusion
	Hgb. (Sahli)	R.B.C.	W.B.C.	Hgb. (Sahli)	R.B.C.	W.B.C.	
	per cent	million		per cent	million		
1	68	3.96	14,600	80	4.40	7,800	Yes
2	72	4.10	16,500	85	5.00	7,400	Yes
3	73	3.90	10,900	95	5.10	6,800	Yes
4	86	5.50	10,500	92	5.18	7,800	No
5	83	5.20	11,900	81	5.00	9,000	No
6	70	4.00	9,000	80	4.00	9,000	Yes
7	72	4.20	14,000	88	4.50	9,200	No
8	68	4.25	10,700	92	4.98	10,000	Yes
9	72	4.08	11,300	75	4.10	8,800	No
10	50	2.70	13,000	82	5.00	7,800	Yes
11	56	2.90	17,800	Died on 4th day			Yes

bers of red blood cells in the urine is the most constant expression of the disorder.

The results are shown in Table V. Briefly, while the intensity of the process as judged by the urinary sediment was usually diminished, the lesions apparently did not heal altogether except perhaps in Case 7 who had very slight changes from the start. Case 2 at autopsy showed only a few scarred glomeruli and it is quite possible that a progressive lesion was arrested by elimination of the streptococcal infection. That this does not always result, however, we have pointed out elsewhere (8). At any rate, long observation with correlation of sediment and histological changes will be necessary in other cases before the final answer is obtained.

Reactions and untoward effects

Complaints of soreness in the buttocks were negligible in spite of the large number of injections which during the course of weeks amounted to several hundred. In a few cases, temperature which had remained slightly elevated became normal when the injections were stopped. This suggests that there was a mild reaction associated with the trauma of frequent hypodermics. In some patients, slight elevations of temperature, or occasionally a sharp spike may have been due to non-specific pyrogenic material. Two patients had transient mild urticarial eruptions. The majority of those who received prolonged intravenous infusions of penicillin developed phlebitis of su-

TABLE V

Effect of penicillin therapy on the renal lesion of bacterial endocarditis as shown by study of urinary sediment

Case no.	Degree of lesion at start of treatment	Degree of lesion at end of treatment	Degree of lesion at last follow-up	Course
1	++++	++	+	Improved
2	++++	++	+	Died—Autopsy: a few scarred glomeruli
3	+++	++	+	Improved
4	0	0	0	
5	0	0	0	
6	+++	++	+	Improved
7	+	0	0	Well
8	++	+	+	Improved
9	++	+	+	Improved
10	++++	++++	No exam.	No change
11	++++			Died—Autopsy

4 plus = Large numbers of R.B.C., white and epithelial cells, many casts, and increase of protein output.

1 plus = A slight increase in output of R.B.C. and W.B.C., possibly a few casts and a trace of protein.

2+3 plus = Intermediate findings.

perforated arm veins which subsided with rest and compresses.

In summary, it may be said that intensive penicillin therapy over long periods of time was unassociated with any toxic effects attributable to the material itself.

Late results

The present report is concerned mainly with the immediate course of events under penicillin therapy even though the follow-up periods extended up to 6 months. Case 11 died on the fourth day of treatment—too soon to draw any conclusions. All the other patients, as evidenced by clinical findings and blood culture, were rendered bacteria-free² and we were inclined to regard them as "cured" (see Table VI). In Case 2, however, the findings at autopsy of cocci in the depths of the "healed" valve lesion makes it necessary to modify any positive statement. Should bacteremia and clinical symptoms recur, one might be dealing with either reinfection or relapse, and a comparison of strains from the original and subsequent attacks would be necessary to settle the matter. Full understanding of the whole situation is obviously contingent on long periods of

² Throughout this paper bacteria-free means that repeated blood cultures, using broth flasks and poured blood agar plates, yielded no growth.

TABLE VI

Summary of results of treatment

	No.	Per cent
Total cases	11	100
Rendered bacteria-free	10*	91
Rendered bacteria-free and clinically "cured" except for old signs of heart lesion	8	73
Clinically cured of active infection after follow-up period of from 2 to 6 months	8	73
Rendered bacteria-free but died later in cardiac failure	1	9
Rendered bacteria-free but signs of cardiac failure later	1	9
Death during treatment (4th day) unexplained	1	9
Relapses or reinfections	0	0

* The one patient not rendered bacteria-free died on the fourth day of treatment. No culture had been made since the second day.

"Bacteria-free" throughout this paper refers to a status in which the blood culture repeatedly yielded no growth.

observation and more autopsies in "apparently healed" cases.

As to functional results, 7 of the 11 patients are back at work and apparently as well as before bacterial endocarditis developed. One patient after a short follow-up period still feels tired and is short of breath on effort although there are no signs of infection, and another, although bacteria-free, now has frank cardiac failure. One patient is dead of cardiac failure (Case 2) and one patient died early in the course of treatment.

There seemed no doubt about the relation of therapeutic effect to strain sensitivity and unless the organism is readily inhibited in the test tube it seems hardly worthwhile starting treatment. A young man had a bacterial infection in connection with a patent ductus arteriosus. A non-hemolytic streptococcus isolated from the blood grew freely in high concentrations of penicillin. Intensive therapy consisting of as much as 500,000 units per day by continuous intravenous infusion failed to lower fever or to reduce the bacteremia. Another patient, a young woman, with all the typical clinical features of bacterial endocarditis, was treated in spite of the fact that numerous blood cultures yielded no growth. There was no benefit and later influenza bacilli were recovered from the blood stream on 3 occasions.

DISCUSSION

With the exception of 1 who died of unexplained causes early in the course of treatment,

10 consecutive patients with bacterial endocarditis with strains of streptococcus sensitive to penicillin were rendered "bacteria-free" and clinically cured of the infection although 1 died later of cardiac failure. Seven, or well over half, are back at work and so far appear to be in as good health as before the infection.

The question comes up as to the explanation of these results in view of the high percentage of failures reported in the early days of penicillin treatment of bacterial endocarditis. We believe that the time factor is paramount and within limits perhaps even more important than the daily dose. Everything indicates that continuous treatment over a long period (2 months or more if necessary) is much more likely to extirpate the infection completely than brief runs of penicillin, even if the daily dose is large. Granting this premise, one wonders whether the technique of administration cannot be simplified. We are now trying an entirely new program consisting from the start of intramuscular injections of 50,000 units of penicillin given only 4 times in the 24 hours (at 6-hour intervals). Three patients now under treatment by this method have responded with prompt fall in temperature and clearing of the blood stream. But long time studies are obviously necessary to settle these questions and, as said above, the present paper deals mainly with the immediate effects of penicillin.

CONCLUSIONS

Intensive penicillin therapy over long periods (approximately 6 to 8 weeks) led to the following results in 11 patients with bacterial endocarditis caused by strains of streptococcus sensitive in the test tube.

1. All patients were promptly made "bacteria-free" (blood cultures) except one who died early in the course of treatment.

2. Eight, or 73 per cent, were clinically cured of the infection after follow-up periods up to 6 months.

3. One patient, apparently cured of the infection, died of cardiac failure. Cocci were seen in the depths of a scarred mitral valve. The significance of this observation is not clear.

4. There were no clinical relapses or reinfections.

5. Petechiae and emboli continued for some time after the blood cultures were negative.

6. Renal lesions as evidenced by studies of urinary sediment were not as a rule completely eliminated by the treatment.

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ANEMIA, HYPOPROTEINEMIA, AND CATARACTS IN SWINE FED CASEIN HYDROLYSATE OR ZEIN. COMPARISON WITH PYRIDOXINE-DEFICIENCY ANEMIA¹

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Certain investigators (1) have recently confirmed the earlier limited data (2 to 4) which indicated that rats maintained on a diet in which the protein was supplied in the form of an acid hydrolysate of casein became anemic and that this anemia was relieved by the administration of tryptophane. The anemia observed by these workers was very slight in degree. The hemoglobin was reduced below 12.5 grams in 10 out of 16 animals but only in 1 of these was it as low as 10.0 grams. In addition, these investigators noted that a reduction in plasma protein preceded the fall in hemoglobin. These findings differ from those of another worker (5) who placed 9 rats on a tryptophane-low diet consisting of equal parts of acid hydrolyzed casein and zein and observed anemia in only 2 of the animals. The former workers criticize the latter's work on the ground that the diet was not entirely free from tryptophane and the experiments were not of sufficiently long duration.

Observations in other species are even more limited. Evidence from experiments in dogs indicates that tryptophane plays a rôle in the formation of plasma proteins (6) and hemoglobin (3, 4, 7). In tryptophane deficiency produced experimentally for periods of 2 to 5 weeks in human subjects, one of us (M. M. W.) working with the first mentioned workers (1) was unable to demonstrate significant alterations in the red cells. The following observations in swine, although also limited in number and in scope, are reported

because of the pronounced anemia and hypoproteinemia which developed when casein in the diet was replaced by acid hydrolyzed casein.

We have also been interested in studying the relationship of the anemia developing in pigs fed diets poor in tryptophane with that seen in pyridoxine deficiency since, in the latter, abnormal metabolism of tryptophane occurs (8, 9, 10). Pyridoxine-deficient swine excrete xanthurenic acid, "kynurenine," and another indole derivative in the urine in abnormal quantities. It therefore seemed desirable to ascertain the effect of low tryptophane intake on the course of pyridoxine deficiency as well as to compare the anemias developing in each type of deficiency.

MATERIAL AND METHODS

Full details of the experimental method have been published elsewhere (11). Pigs were obtained from the Bureau of Animal Industry, the United States Department of Agriculture, Beltsville, Maryland, except animals 8-01 to 8-05, inclusive, which were obtained from a private source. These animals were of Chester White breed and suckled until approximately 3 weeks of age, when the experiment was commenced.

The hydrolysate of casein³ was an acid hydrolysate and by analysis was free of tryptophane. When supplemented with tryptophane, it permitted growth of at least 10 grams per week in rats.

The standard diet consisted of casein,⁴ 26.1 per cent; sucrose, 57.7 per cent; lard, 11.0 per cent; swine salt mixture no. 3 (11), 5.2 per cent; 36.4 grams of this mixture constitutes one "kilo unit" and furnished 152 calories. When the casein was replaced by casein hydrolysate or zein,⁵ it is so stated in the tables and protocols. When less than 26 per cent casein or casein hydrolysate was used, the caloric deficit was made up

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³ Products 89-9 and 89-10, prepared by Mead Johnson and Company from HIP quality casein of the Casein Corporation of America.

⁴ Sheffield "New Process," Sheffield By-Products Company.

⁵ Corn Products Company.

with sucrose. In addition, the animals received cod liver oil⁶ (1800 units A, 175 units D per gram), 0.5 gram per kgm. body weight daily. Vitamins⁷ were supplied in crystalline form by placing them in capsules and administering them orally. The quantities of crystalline vitamins given were as follows (mgm. per kgm. body weight daily): thiamin hydrochloride, 0.25; riboflavin, 0.12; nicotinic acid, 1.20; pyridoxine hydrochloride, 0.20; calcium pantothenate, 0.50; p-aminobenzoic acid, 0.50; inositol, 1.20; choline chloride 10.0. The animals were fed the above standard diet supplemented with all 8 vitamin supplements for 1 week. After this time, the appropriate protein substitutions were made and the pyridoxine hydrochloride was omitted from the vitamin supplements given the groups which were to become pyridoxine-deficient. All animals receiving acid hydrolyzed casein took the diet poorly when it was first offered and, for this reason, for the first 2 weeks of the experiment, animals 7-98 and 7-99 were given half casein and half casein hydrolysate while pigs 8-01 and 8-02 were given half zein and half casein hydrolysate.

Serum iron determinations were made by the method of McKibbin *et al.* (12) by precipitating 5 milliliters of serum with trichloroacetic acid at 90° C. The filtrate was then adjusted to pH 6 with ammonium hydroxide and the iron determined with alpha-alpha dipyridyl after reducing with thioglycolic acid. A correction factor was used for the amount of iron which was carried down by the protein precipitate. Serum iron determinations by this method are accurate within ± 10 per cent. Great care was taken in cleaning glassware and in preparing reagents free of iron.

Xanthurenic acid was measured presumptively and qualitatively by neutralizing the urine to litmus, adding a few drops of ferric ammonium sulfate and filtering (8). The depth of the green color was then noted.

Serum non-protein-nitrogen was determined by the method of Dr. M. V. Buell which makes use of a persulfate digestion followed by nesslerization in the presence of potassium gluconate. This is read in the photoelectric colorimeter at wave length 500. Total serum protein was determined by a macro Kjeldahl analysis as adapted by Dr. Buell in which selenium is used as a digestant and the ammonia collected in boric acid. Albumin and globulin separations were performed by the method of Kingsley (13) and then measurements were made by the macro Kjeldahl method.

RESULTS

Groups I and II—"Acid Hydrolyzed Casein" and "Control"

Group I consisted of 3 pigs (7-98, 7-99, and 7-05), while Group II consisted of only 1 animal (7-03). This animal was fed the casein used in making the acid hydrolysate for 7-05 and was

given that amount of diet which 7-05 consumed the day preceding. In this manner, it served as an inanition control. Both animals were given a diet containing 26 per cent casein and received in addition a supplement of 0.07 gram cystine per kgm. body weight per day. The 2 remaining animals of Group I were fed a diet containing only 15 per cent acid hydrolyzed casein. The results are summarized in Table I.

The animals receiving acid hydrolyzed casein ate poorly, failed to show any appreciable growth, and died on the 74th, 115th, and 117th days of the deficiency. Definite anemia developed which was normocytic, or slightly microcytic, and normochromic. The anemia appeared early and was slowly progressive. There was no evidence of increased hemolysis as determined by icteric index and qualitative urobilinogen determinations. The serum iron levels remained at normal limits even at the height of the anemia and there was no reticulocytosis. Terminally, that is a week or less prior to death, leukopenia appeared and persisted in all 3 animals. Differential leukocyte counts, however, revealed no consistent or significantly greater reduction in the number of cells of one series of leukocytes as compared with another. Blood platelets were not reduced in number.

These animals also developed pronounced hypoproteinemia with reduction of both albumin and globulin. The hypoproteinemia and anemia appeared to develop simultaneously. The results of Tiselius electrophoretic studies are given in Table II.⁸ For comparison with these determinations, the blood serum of a pig (7-94) fed the standard diet in reduced amounts ("inanition control") was also examined. The globulin fractions are designated as proteins Nos. 2, 3, and 4 since these appeared in slightly different arrangement as compared with those of human serum. The most pronounced change was in the albumin fraction which showed a marked reduction and some change from normal composition as indicated by a broad asymmetrical curve.

When the blood changes described above had become quite pronounced, pig 7-98 was given 0.5 gram *d,l*-tryptophane daily (Figure 1). The animal died 18 days later. This small amount of

⁶ Mead Johnson and Company.

⁷ Merck and Company.

⁸ We are indebted to Dr. John Lusterer, Jr., of the Chemical Division for these determinations.

TABLE I
Summary of the data on blood studies

Group:	I			II	III		IV			V			VI
Protein, type	Acid hydrolyzed casein			Ca-sein	Acid hydrolyzed casein		Zein			Casein			Ca-sein
Protein, per cent	15	15	26	26	18		18			26			26
Vitamin supplement	Complete			Complete	Pyridoxine omitted		Pyridoxine omitted			Pyridoxine omitted			Complete
Animal number	7-98	7-99	7-05	7-03	8-01	8-02	8-03	8-01	8-05	7-80	7-82	7-84	
Duration experiment, days	117	115	74	74	69	46	59	64	90	66	78	73	66
Termination ¹	D	D	D	K	D	D	D	D	D	K	K	K	K
RBC, millions per c. mm.	3.87	4.36	5.53	7.80	4.80	6.20	5.60	3.12	3.86	5.25	6.40	8.30	7.93
Hemoglobin, grams per cent	6.8	8.6	9.3	16.7	7.7	9.3	9.0	5.4	5.9	6.1	8.4	10.1	14.0
Volume packed red cells, ml. per 100 ml.	20.4	24.0	27.6	49.0	25.7	29.5	29.2	17.6	20.2	21.6	27.8	34.5	46.0
MCV ² , cubic microns	53	55	50 ³	63 ³	53	48	52	56	53	41	43	42	58
MCH ² , micrograms	18	20	17	21	16	15	16	17	15	12	13	12	18
MCHC ² , per cent	33	36	34	34	30	32	31	31	29	28	30	29	33
WBC, thousands per c. mm.	5.8	6.9	4.0	13.8	6.4	6.3	14.0	12.4	10.3	12.2	16.6	18.5	18.0
Serum iron, µg. per cent	143	120			145	144	116	72	75	422	465	370	142
Serum NPN, mgm. per cent	23	31	50	42	32	31	23	18	32	32	23	29	19
Total serum protein, grams per cent	2.81	4.44	4.41	6.97	4.38	4.06	3.19	2.63	3.00	6.44	6.63	6.31	6.33
Albumin, grams per cent	1.38	2.44	2.05	3.19	2.88					4.06	4.00	4.12	3.69
Globulin, grams per cent	1.43	2.00	2.36	3.78	1.50					2.38	2.63	2.18	2.64
Albumin-Globulin ratio	0.92	1.22	0.87	0.85	1.92					1.71	1.52	1.89	1.39

¹ D indicates that the animal died, K that it was sacrificed.

² MCV refers to mean corpuscular volume; MCH, mean corpuscular hemoglobin; and MCHC, mean corpuscular hemoglobin concentration.

³ The mean red cell diameter at this time was 5.09 microns in pig 7-05 and 5.48 µ in pig 7-03.

tryptophane had no effect on the anemia and there was no reticulocytosis. However, the total serum protein rose from 2.81 grams per cent to 4.19 grams per cent.

One of these animals developed ocular lens opacities. When the experiment had been in progress 80 days, a fine, somewhat wavy line of opacity was noted near the equator of the lenses of pig 7-98. A week later opacities were noted along the anterior suture lines and also at the posterior suture lines. In another week, the anterior suture line opacities were quite pro-

nounced, and posterior capsular or subcapsular opacities as well as peripheral equatorial opacities were clearly visible, especially in the right eye. By this time, the hypoproteinemia and anemia were quite severe. In pig 7-99, only several small vacuoles located nasally below and near the equator of the lens were seen. The hypoproteinemia in this animal was never as pronounced as in 7-98. Pig 7-05 was not found to have cataracts.

Neither epileptiform convulsions nor ataxia, such as occur in pyridoxine-deficient swine (14), were observed in these animals but they became very weak. The gait of 7-05 was described as "staggering" in character and in all the animals the muscular development was very poor. They were very thin and the hair coats were untidy. The hair came out very readily. Edema was present, especially in pig 7-98, where it appeared as a swelling in the under portion of the neck.

At autopsy the tissues were edematous. Histologically the muscle of 7-98 showed large areas where muscle fibers were atrophied or had completely disappeared (Figure 2). Such areas were very cellular. The nuclei resembled those of

TABLE II
Tiselius electrophoretic analysis of serum proteins of pigs fed casein hydrolysate

Condition	Animal number	Total serum protein	Albumin fraction	Globulin fraction			
				Total	No. 2	No. 3	No. 4
		grams per cent	grams per cent	grams per cent			
Inanition control casein, 15 per cent	7-94	5.00	2.42	2.58	1.11	0.64	0.83
Casein hydrolysate, 15 per cent	7-98	2.81	0.83	1.98	1.08	0.33	0.57
Casein hydrolysate, 15 per cent	7-99	4.69	1.87	2.82	1.36	0.67	0.79

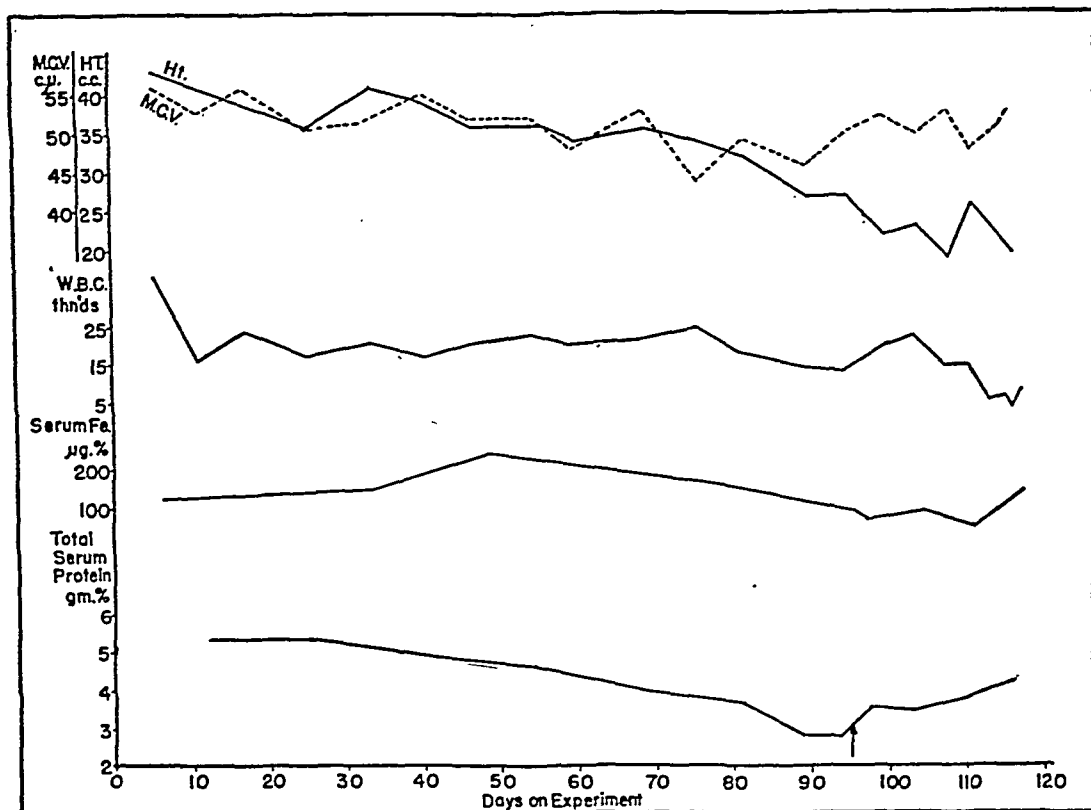


FIG. 1. DEVELOPMENT OF NORMOCYTIC ANEMIA AND HYPOPROTEINEMIA, AS WELL AS TERMINAL LEUKOPENIA, IN A PIG (7-98) FED ACID CASEIN HYDROLYSATE IN THE PLACE OF NATURAL CASEIN

Note the increase in total serum protein following administration of a small quantity (0.5 gram) of *d,l*-tryptophane (arrow). At no time was there a significant rise in serum iron.

MCV refers to mean corpuscular volume; Ht to volume of packed red cells, ml. p. 100 ml. blood. The leukocyte count (WBC) is expressed in thousands per c. mm.

sarcolemma and connective tissue cells. There were a few hyaline muscle fibers but on the whole the changes were not fresh. Muscle from 7-99 showed extreme atrophy and a few fresh focal necroses. In the muscle of pig 7-05, one lesion consisting of a focus of several hyaline muscle fibers was found. Muscle tissue from 7-03 showed no abnormalities.

The bone marrow was pale and, in 7-98, was hypoplastic on microscopic examination (Figure 4). There was no hemosiderosis in the spleen, liver, or bone marrow and no fatty changes were found in the liver. These data are summarized in Table III.

Groups III and IV—"Acid Hydrolyzed Casein, Pyridoxine-Deficient," and "Zein, Pyridoxine-Deficient"

The purpose of these 2 groups was to ascertain the effect of low tryptophane intake on the course

of the pyridoxine deficiency. Pyridoxine was omitted from the vitamin supplement of both groups. Group III consisted of 2 animals receiving a diet containing 18 per cent acid hydrolyzed casein and group IV consisted of 3 animals given a similar proportion of zein in the place of casein. All animals failed to grow and died between the 46th and 90th day of the experiment. A moderately severe anemia developed which was normocytic, or slightly microcytic, and normochromic. The 2 animals of group III developed a terminal leukopenia but the animals maintained on zein failed to show this. As in group I, there were variations in the differential leukocyte counts but no significant alterations could be correlated with the changes in the total count. The serum iron remained within normal limits. There was a pronounced reduction in total serum protein.

Epileptiform convulsions or staxia were not observed. Furthermore, no xanthurenic acid

could be demonstrated in the urine of these animals. As in the case of the pigs of group I, weakness and edema characterized these animals but in the pigs fed zein in the place of casein (group IV), edema was much more pronounced. This was especially noticeable about the eyelids, the under part of the neck and the genitalia (Figure 3). The swelling about one eye of pig 8-04 was so great that the eyeball was hidden. Post-mortem examination revealed extreme edema of all the subcutaneous tissue, and in pig 8-05, there was serous fluid in the abdominal cavity.

The bone marrow of pigs 8-01 and 8-02 was slightly to moderately hyperplastic (Table III). A slight to moderate degree of hemosiderosis was present. These changes seemed less pronounced than we would have expected in animals not given pyridoxine for the same length of time, as judged by past experience (14). The findings in pigs 8-03, 8-04, and 8-05 (Figure 6) were similar. These last 3 animals had received pyridoxine, 200 micrograms intravenously daily, respectively, for the 7, 3, and 8 days preceding the autopsies.

The muscle fibers in pigs 8-01 and 8-02 were very atrophic. No necroses were found. In pigs 8-03 and 8-05, there were a few small foci consisting of extremely atrophic hyaline muscle fibers surrounded by mononuclear cells. Pig 8-04 was found to have extreme atrophy with hyalinization of the muscle fibers so that virtually no transverse striations could be found. Whether due to the decrease in muscle tissue or to a true proliferation of sarcolemma nuclei, the tissue was very cellular.

There were no lens changes in pig 8-03. Animal 8-04 developed in the lens of the right eye

peripheral cortical opacities above and temporally, as well as lens fiber dissociation and one group of vacuoles nasally. In the lens of the left eye, there was 1 group of vacuoles in the peripheral cortex nasally and there were 2 patches of opacities temporally and above. These were possibly also in part vacuolized. In pig 8-05, when the experiment had been in progress 37 days and the serum proteins were 4.19 grams per cent, very marked cortical lens fiber dissociation was noted in both eyes and a small number of groups of vacuoles were visible near the equator. As the deficiency progressed, the lens fiber dissociation became more pronounced and after another 23 days both lenses showed advanced posterior capsular cataracts, particularly around and at the posterior pole. Sixteen days later there was a very outspoken posterior rosette-type opacity in each eye and a streaky appearance throughout the lenses. The changes in the right eye were more pronounced than in the left eye.

Groups V and VI—"Pyridoxine-Deficient" and "Normal Controls"

Group V consisted of 3 animals fed the standard diet containing 26 per cent casein but with pyridoxine omitted from the vitamin supplements. These animals have been considered elsewhere (9) and the data are presented here only for comparison. By the 66th day of the deficiency, these animals had developed microcytic anemia and manifested epileptiform convulsions from time to time. The urinary test for xanthurenic acid was positive. Serum iron levels were greatly elevated but there was no reduction in total serum protein and no evidence of edema.

PLATE I

FIG. 2. MUSCLE FROM TONGUE OF PIG (7-98) FED ACID HYDROLYSED CASEIN.

Note normal muscle fiber and hyaline necrotic ones. The latter are infiltrated by leukocytes, mostly mononuclears. There are also occasional giant cells. Areas such as these were focal and diffuse.

FIG. 3. MARKED EDEMA IN A PIG (8-04) FED ZEIN IN THE PLACE OF CASEIN AS A SOURCE OF PROTEIN.

Note especially the edema in the neck and about the penis. The total serum proteins at this time were 2.63 grams per cent (normal over 6.00 grams).

Figures 4, 5, and 6. Bone Marrow. Custer Stain, $\times 100$

FIG. 4. PIG (7-98) FED ACID HYDROLYSED CASEIN. There is no hyperplasia. (The dark areas between the fat cells contain no marrow cells.)

FIG. 5. PRONOUNCED HYPERPLASIA IN THE MARROW OF A PIG (7-82) FED A DIET DEFICIENT IN PYRIDOXINE.

FIG. 6. PIG 8-05 FED ZEIN AND ALSO NO PYRIDOXINE UNTIL 8 DAYS PRECEDING AUTOPSY WHEN 200 MICROGRAMS WERE GIVEN DAILY BY VEIN.

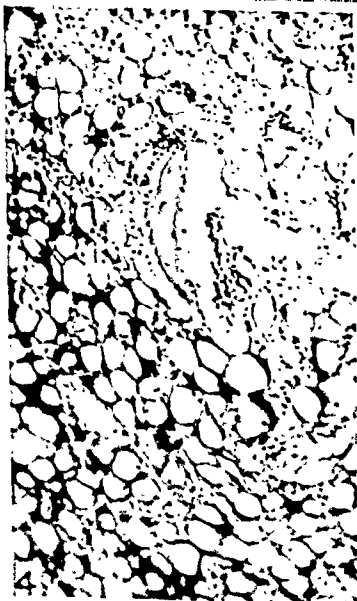
There is very minimal hyperplasia, as would be expected following treatment with pyridoxine.



2



3



4



5



6

PLATE I

TABLE III
Summary of clinical and pathological studies

Group	I			II	III		IV			V			VI
Protein, type	Acid hydrolyzed casein			Casein	Acid hydrolyzed casein		Zein			Casein			Casein
Protein, per cent	15	15	26 ¹	26 ¹	15	15	18	18	18	26	26	26	26
Vitamin supplement	Complete			Complete	Pyridoxine omitted		Pyridoxine omitted			Pyridoxine omitted			Complete
Animal number	7-98	7-99	7-05	7-03	8-01	8-02	8-03	8-04	8-05	7-80	7-82	7-84	1.0
Average daily food intake, units ²	0.7	0.6	0.6	0.6	0.6	0.6	0.9	0.8	0.9	0.9	0.9	1.0	1.0
Total weight gain or loss, kgm.	+1.3	+0.5	-2.4	+6.7	-0.1	-0.6	+1.0	+1.2	+0.9	+10.6	+13.1	+4.0	+12.0
Ataxia	0	0	0	0	0	0	0	0	0	+	+	+	0
Convulsions	0	0	0	0	0	0	0	0	0	+	+	+	0
Edema	++	+	+	0	++	++	++	++	++	+	+	+	0
Bone marrow, hyperplasia ³	0	0	0	+	±	+	+	±	+	+	+	+	0
Hemosiderosis liver	0	0	0	0	0	+	0	+	0	0	0	0	0
Spleen, pulp	0	0	0	0	+	++	0	++	++	++	++	±	0
trabeculae	0	0	0	0	0	0	0	0	0	++	++	0	0
Bone marrow	0	0	0	0	0	0	0	0	0	0	0	0	0
Fatty liver	0	0	0	0	0	0	+	++	0	+	+	0	0
CNS degeneration	0	0	0	0	0	0	0	0	0	++	++	+	0
Muscle degeneration	++	++	+	0	++	++	+	++	+	+	+	+	0
Lens opacities	+	+	0	0	0	0	0	+	+	0	0	0	0

¹ Plus cystine 0.07 grams per kgm. per day.

² One "unit" is an amount of the dietary mixture furnishing 152 calories per kgm. body weight daily.

³ Under Bone Marrow Hyperplasia, + indicates "moderate," ± "slight," 0 "none," and 0 "hypoplastic."

⁴ Pigs 8-03, 8-04, and 8-05 were given intravenously 200 micrograms pyridoxine per kgm. body weight daily during their 7, 3, and 8 last days, respectively.

⁵ The degenerative changes in the nervous systems of these animals were found only in the peripheral nerves.

⁶ Muscle tissue was not examined in these animals but in numerous other pyridoxine-deficient pigs which have been examined no degeneration has been encountered.

⁷ This was periportal rather than central, as occurs in pigs deficient only in pyridoxine.

There was no terminal leukopenia. When these animals were sacrificed, no edema could be demonstrated at autopsy. There was hyperplasia of the bone marrow (Figure 5), hemosiderosis of the spleen, the livers showed central fatty infiltration, and there were demonstrable changes in the central nervous system. No cataracts were observed in these pigs.

Group VI consisted of a large number of control animals receiving the standard diet containing 26 per cent casein, and fed a complete supplement of the 8 "B" vitamins listed under Material and Methods. Average findings are shown in Table I. In such animals, we have found no reduction in hemoglobin or serum proteins. At autopsy, the edema and muscle necrosis, the malnutrition, the bone marrow hyperplasia, and the hemosiderosis described in groups I, III, IV and V, were not encountered.

DISCUSSION

Circumstances have made it impossible to extend these studies to a larger series of animals and to include the various experimental groups which would be desirable in a complete experiment. Nevertheless, it seems clear from these data that in swine, the feeding of a diet composed of acid hydrolyzed casein instead of the natural

product, but presumably adequate in other respects, is associated with the development of marked hypoproteinemia and anemia. This occurred even when the hydrolyzed casein was fed at a 26 per cent level rather than 15 per cent. The anemia was normocytic or slightly microcytic but the concentration of hemoglobin in the red corpuscles remained unchanged. The hypoproteinemia, which was due more to a reduction of albumin than of globulin, was associated with the development of edema. At the same time, there were failure to gain weight, focal atrophy and necrosis of muscles, and cataracts in the lenses of the eyes.

The pigs fed 18 per cent zein in the place of acid hydrolyzed casein were also given no pyridoxine, but comparison with pyridoxine-deficient animals fed natural casein suggests that the changes associated with the feeding of a zein diet are similar to those associated with the feeding of acid hydrolyzed casein. In fact the hypoproteinemia and edema were even more pronounced.⁹

⁹ This greater degree of hypoproteinemia may have been due to the effect of lysine deficiency in addition to tryptophane deficiency. Lysine deficiency has been shown recently to be associated with the development of hypoproteinemia (Harris, H. A., Neuberger, A., and Sanger, F., *Biochem. J.*, 1944, 37, 508).

The most striking difference between the findings in the pigs fed acid hydrolyzed casein and in those fed zein was the development of leukopenia terminally in the former.

The presence of lens changes in pigs receiving a diet furnishing acid hydrolyzed casein or zein instead of natural casein, confirms the findings of others (15 to 18). Certain investigators (17, 18) reported that rats maintained on a diet deficient in tryptophane (acid hydrolyzed casein) developed cataractous changes. These changes could be prevented by supplementing the diet with tryptophane. Also described (17) were cataractous changes in rats maintained on a diet of zein. These changes were not influenced by the concurrent lysine deficiency but could be prevented by feeding tryptophane.

The fact that similar changes took place in our animals when they were maintained on acid hydrolyzed casein or on zein, suggests that these effects were due to a lack of tryptophane rather than to lysine which zein lacks in addition to being deficient in tryptophane. We can offer no direct proof of this as sufficient tryptophane was not available to determine whether such a supplement to the diet would completely restore the animals to normal. That the changes were not due to inanition is indicated by the fact that animal 7-03 was fed the same quantity of a complete diet as 7-05 consumed of the deficient one. Pig 7-03 nevertheless failed to develop either anemia or hypoproteinemia.

That a disturbance in tryptophane metabolism occurs in pyridoxine deficiency is indicated by the fact that pyridoxine-deficient swine excrete xanthurenic acid, "kynurenine," and at least one other indole derivative in abnormal quantities (9, 10). Since either a lack of tryptophane or of pyridoxine leads to the development of anemia, the question arises whether pyridoxine anemia might indirectly be due to a lack of properly metabolized tryptophane. If such were true, the 2 anemias should be similar.

The 2 types of anemia, however, are very different from one another. The anemia of pyridoxine deficiency is microcytic and slightly hypochromic and is accompanied by an elevated serum iron, hyperplastic bone marrow, and hemosiderosis of the spleen, liver, and bone marrow. The anemia associated with the feeding of acid hydro-

lysed casein and presumably due to tryptophane deficiency is essentially normocytic and normochromic, the bone marrow appears to be hypo- or normo-plastic, the serum iron level is normal and there is no hemosiderosis of the tissues. Furthermore, the 2 deficiencies are quite different clinically. "Tryptophane" deficiency causes cessation of growth and is accompanied by marked hypoproteinemia and edema. Pyridoxine deficiency causes only limitation of growth and the quality and quantity of the serum proteins are unaffected.

It is of interest to speculate in what manner tryptophane, pyridoxine, and iron are utilized in blood formation. A simple explanation of the rôle of these substances is that union of tryptophane, iron, and other factors (X) takes place ($T + Fe + X \rightarrow RBC$), with pyridoxine acting as an essential component of an enzyme system promoting this reaction. When T is lacking, Fe is still bound to X, the enzyme being present, and no ferremia occurs. Anemia develops, however, because T is lacking. When Fe is lacking, T is still bound to X, since the enzyme is present. No ferremia occurs and products of tryptophane metabolism are not excreted although anemia occurs owing to iron deficiency. We have found (9) these to be the actual facts in pigs fed iron-poor diets. On the other hand, when the theoretical enzyme system involving pyridoxine is lacking, no union of the building stones takes place. As a result, ferremia occurs and substances derived from tryptophane are lost in the urine. If this hypothesis is correct, it should be expected that feeding tryptophane to pyridoxine-deficient animals will result in increased excretion of xanthurenic acid. This has actually been found to be the case (8, 9). It may be that other substances, derived from X, are also excreted. The theory presupposes that total body iron is normal in pyridoxine deficiency, which the obvious hemosiderosis would appear to deny. In current experiments, attempts are being made to study this question.

It is interesting that, although the degree of anemia is much the same, the bone marrow in pyridoxine deficiency is hyperplastic whereas the marrow of the pigs maintained on acid hydrolysate of casein was normo- or hypoplastic. The factors governing the cellularity of the bone mar-

row are unknown. Certainly anemia *per se* is not the only governing factor. It is recognized that anemia occurs in the presence of hyperplastic, normoplastic, or hypoplastic marrow.

Our results, although very limited, suggest that the development of pyridoxine deficiency may be retarded when the tryptophane intake is decreased. The pyridoxine-deficient animals fed casein acid hydrolysate and those given zein failed to develop certain signs of pyridoxine deficiency in 46 to 90 days, namely: pronounced microcytosis, elevated serum iron, epileptiform convulsions, and ataxia. Histologic evidence of changes in the nervous system was also lacking. Hemosiderosis was not as marked as might have been expected in pyridoxine deficiency of this duration. Pyridoxine-deficient pigs, started on experiment at a similar age and fed 26 per cent casein, have shown these changes in 4 to 10 weeks (14). These results are in harmony with those of others (19) who studied pyridoxine deficiency in rats fed diets containing 15, 30, and 45 per cent casein. They found that at the low level of protein intake, little dermatitis developed in 70 days, while, at the intermediate level, rats developed dermatitis after 30 days. At a protein level of 45 per cent casein, severe dermatitis developed in 26 days and was followed shortly by death. Apparently, high protein intake in rats seemed to increase the severity of the nutritional disorder due to inadequate intake of pyridoxine. Further studies by these investigators (20) revealed that tryptophane delayed the onset of symptoms and prolonged the life of the animals, whereas cystine aggravated them.

Whether tryptophane is ever the limiting factor in the formation of hemoglobin in humans is not known.

The changes observed in the muscles of the animals fed acid casein hydrolysate or zein in the place of casein, will require much further study. In one pig, there were extensive lesions in which there were atrophy and absence of fibers. Such areas contained very cellular tissue which probably arose from sarcolemma cells. In all the animals, the muscle fibers were extremely atrophic. The fibers in some were hyaline.

SUMMARY

1. Swine maintained on a synthetic diet, in which the protein was supplied in the form of an

acid hydrolysate of casein or by feeding zein, failed to grow and developed normocytic, or slightly microcytic, normochromic anemia which was accompanied by a normal serum iron level, hypo- or normo-plastic bone marrow, and marked hypoproteinemia and edema.

2. Lenticular opacities developed in 2 out of 3 animals maintained on acid hydrolysed casein and in 2 of 3 pigs maintained on zein.

3. It is thought most likely that these changes were caused by a deficiency of tryptophane.

4. Although faulty tryptophane metabolism occurs in pyridoxine deficiency, comparison between "tryptophane" and pyridoxine anemia reveals marked differences. A hypothesis is offered to explain the rôle of tryptophane and pyridoxine in hematopoiesis.

5. The data presented suggest that a low intake of tryptophane retards the course and diminishes the severity of the nutritional disorder due to pyridoxine deficiency in swine.

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PROGRESSIVE ADDISONIAN PERNICIOUS ANEMIA, SUCCESSFULLY TREATED WITH INTRAVENOUS CHOLINE CHLORIDE

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Sudden failure of an occasional patient afflicted with Addison-Biermer's disease (Addisonian pernicious anemia) to respond to adequate amounts of purified liver extracts in the absence of factors known to interfere with a therapeutic response has been observed by us during the past few years in the Minneapolis General Hospital. The occurrence of a refractory state for no apparent reasons has also been noted by others (1 to 4). Because little is known about the chemical nature of the liver factor (anti-pernicious anemia factor) or the physiologic dose, including the development of a progressive anemic state under adequate amounts of purified liver extracts, we felt justified in publishing an observation which we believe may indicate one of the mechanisms responsible for a sudden refractory behavior of certain patients. We could demonstrate in a male afflicted with Addisonian pernicious anemia that fatty liver and fatty metamorphosis of the bone marrow organ were apparently factors in the development of a progressive anemic state.

Without further administration of liver extract, the progressive anemic state was brought into remission by means of the administration of a 5 per cent solution of choline chloride (Merck), 20 ml. intravenously for 16 successive days. The doses were given at a rate of 1 ml. per minute. The first dose caused flushing and some nausea after 10 ml. were administered. No significant drop in blood pressure was observed. The successive doses were well tolerated.

CASE HISTORY

The patient, a male, who for several years responded in a characteristic manner to the administration of liver extracts intramuscularly and maintained a normal peripheral blood status on 3 ml. monthly of fairly crude liver extracts containing about $\frac{1}{2}$ to 2 U.S.P. units per ml., developed a progressive anemic state when purified liver extracts were substituted containing about 10 and 15 U.S.P. units per ml. The peripheral erythrocyte quantity, formerly ranging between 5 and 5.6 mill. per

c. mm. under crude liver extract therapy, gradually dropped within 3 months to a level ranging between 3.5 and 4.0 mill. per c. mm. on 20 to 45 U.S.P. units per month. The patient then developed an increasing sensitivity to purified liver extracts and all attempts to desensitize him by means of procedures recommended in the literature met with failure (5, 6). At this time also, the crude extract, as well as oral liver extract, produced the same allergic symptoms as did the purified liver extracts. The development of a progressive jaundice, several days after the administration of 3 ml. of a purified liver extract, given by error, led to his transfer from our dispensary to the hospital for a general work-up.

This 61-year-old white male, who was hospitalized twice before because of classical Addisonian pernicious anemia in severe relapse responding characteristically to purified liver extracts, entered the hospital this time with the complaint of itching, nausea, vomiting, and jaundice for 2 days following the intramuscular injection of 30 U.S.P. units. On the fifth day, he noted dull aching pain in the right upper quadrant. He passed dark brown urine and light colored stools, and the icterus of the skin became progressively deeper. Physical examination revealed a marked hypertension (B.P. 220/150) and a markedly yellow skin and sclera. The liver extending about 6 cm. below the costal margin was smooth with a sharp edge and non-tender. There were no other essential findings. Routine laboratory findings were as follows: hemoglobin 13.5 grams (Newcomer) = 85 per cent, erythrocytes 3,870,000; color index 1+; white blood cells 5100; reticulocytes 0.5 per cent. The peripheral blood showed neutrophils 46 per cent; lymphocytes 39 per cent; monocytes 11 per cent; eosinophiles 3 per cent; basophiles 1 per cent. A few megalocytes and a small number of poikilocytes were observed. Thrombocytes numbered 140,000 per c. mm. The mean corpuscular diameter (Halometer) was 8.6 microns. Icteric index was 105. The Van den Bergh test, direct and indirect, was positive. Prothrombin time for the patient was 40 seconds, for the control, 24 seconds. Wassermann, Kahn, and Rytz tests were negative. Plasma proteins were: total 8.56; albumin 5.0; globulin 3.01; and fibrinogen 0.55 grams per cent. Urine showed a specific gravity of 1.021, albumin 1+, erythrocytes 4 to 5, leukocytes 1 to 2 per high-powered field, and numerous casts. Roentgenologic examinations of the gastrointestinal tract were negative. Lipemia of the plasma could not be demonstrated.

Needle liver biopsy was performed and several specimens from various areas were obtained. The histologic

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diagnosis was acute catarrhal jaundice and moderate severe fatty liver. (Figure 1-B). Simultaneously with the liver biopsy, sternal marrow was aspirated from the proximal end of the body of the sternum at the level of the second interspace and worked up by one of us (E. M. S.), according to methods published elsewhere (7, 8). The smear preparations made from the aspirated marrow tissue showed that the marrow organ was moderately severely megaloblastic, indicating that a deficiency of the anti-pernicious anemia factor existed. The gross marrow units (marrow particles) were numerous, varied in size, and ranged between 1 and 4 mm. (normal 0.5 to 1 mm.), suggesting that the marrow organ was

either hypertrophic or the individual units contained an excess amount of fat. The heparinized marrow specimen was transferred into a Wintrobe hematocrit tube and centrifuged at 1500 R.P.M. per minute for 5 minutes. The following layers were obtained from the top of the tube down: fat 10; plasma 30; nucleated cells or immature myeloid and erythroid cells (M-E) 17; mature erythrocytes derived from ruptured sinusoidal-blood vascular systems (Er.) 48 volumes per cent.²

² The normal range of the sternal volume percentage of the adult male as determined by one of us (E. M. S.) is believed to be: fat 0.5 to 3.0; plasma 39 to 48.5; M-E 4 to 6; Er. 45 to 54.

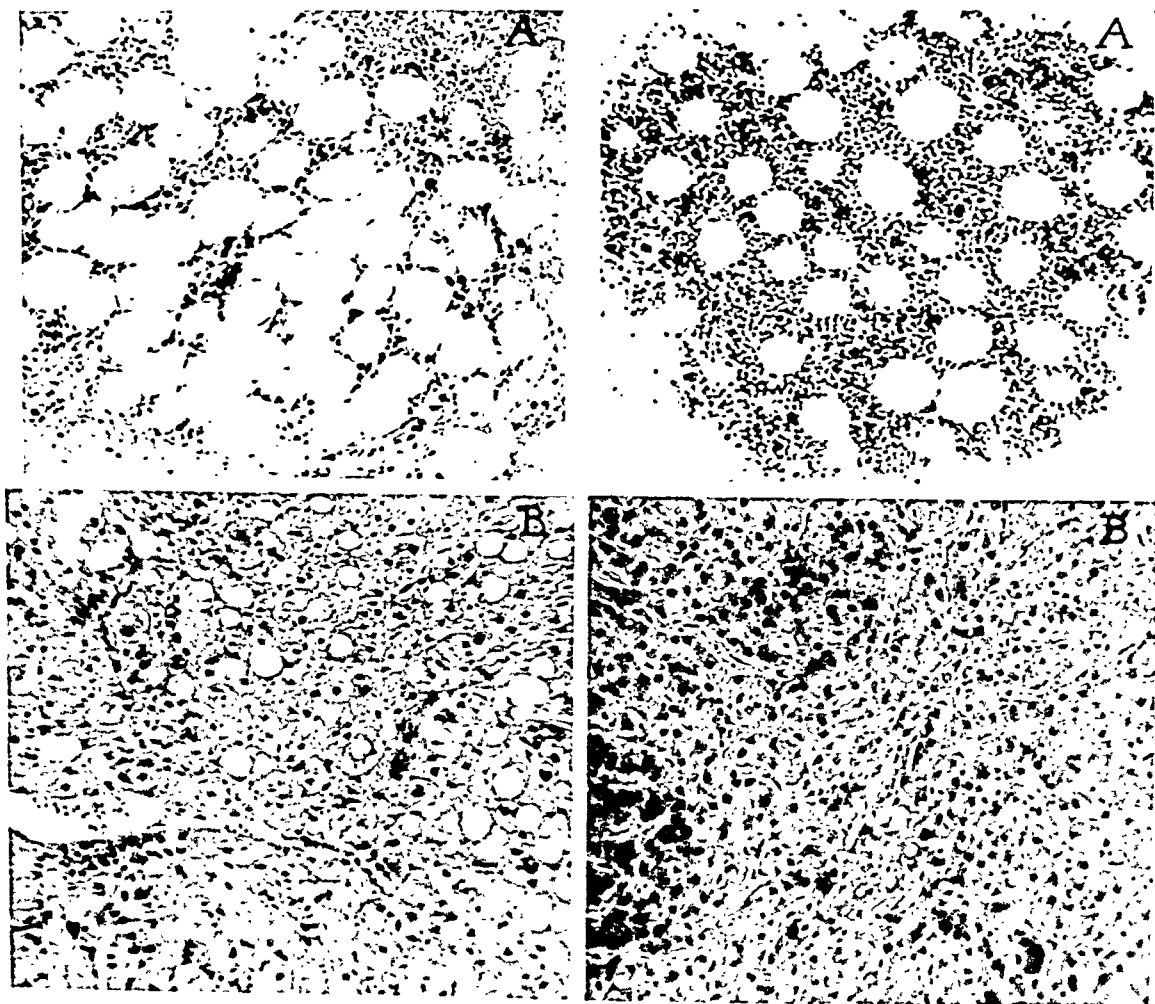


FIG. 1.

A. Average microscopic marrow unit (sternum) before choline therapy showing hypoplasia and severe fatty metamorphosis.

B. Liver (needle biopsy) before choline therapy showing fatty metamorphosis and pattern of catarrhal jaundice.

FIG. 2.

A. Average microscopic marrow unit (sternum) after choline therapy showing hyperplasia and a normal maximum number of fat spaces.

B. Liver (post-mortem) showing fewer fat cells and polymorphonuclear neutrophils.

scopic marrow units (bounded by 25 squares of the Whipple ocular micrometer when the following optical system is used, eyepiece 10 \times and objective 10 \times , showed conspicuous fatty metamorphosis and a moderately severe hypoplasia of the hematopoietic tissue. The number of fat spaces ranged between 45 and 60 per 25 squares.³

Figure 1-A shows an average microscopic unit with fatty metamorphosis and hypoplasia. Generally the microscopic marrow units in uncomplicated cases are hyperplastic and show none to a few fat spaces. On the basis of the fatty liver and marked increase of the fat in the marrow organ, both macroscopic and microscopic, one of us (E. M. S.) advanced the hypothesis that the fatty state was the factor responsible for the development of the progressive anemic state in our patient and that the fatty state may have resulted from a choline insufficiency. The choice of choline and dosage for reducing the fat deposit in the liver and bone marrow organ were influenced by several factors. First, by the observations of others (9 to 14) that choline is a lipotropic substance important in the mechanism of fat transportation and deposition. Second, on the data accumulated by one of us (E. M. S.) on the lipotropic effect of daily intravenous injection of 10 ml. of a 1 per cent choline chloride solution (Merck) over a period of from 5 to 10 days on the fat volume percentage of the sternal part of the adult human bone marrow organ of cases with cirrhosis of the liver (confirmed by needle biopsy) and carcinoma of the stomach, with and without metastases to the bone marrow. These data showed that a 1 per cent solution reduced excess marrow fat to a normal volume percentage within a few days without causing alarming symptoms. No appreciable changes of the peripheral blood status were observed. And third, the daily dosages were determined on the basis of work done by one of us (F. B. M.) relating to the effect of choline chloride on the blood pressure of patients with essential hypertension.

Figure 3 shows the peripheral blood status before, during, and after choline chloride therapy and the fat volume percentage of the bone marrow organ before and at the time therapy was discontinued. As can be easily seen, there was no appreciable change of the peripheral blood status during a 12-day pre-treatment period. Administration of choline chloride (Merck) was followed by an increase of reticulocytes reaching a peak of 5.5 per cent on the third day. The obtained reticulocyte peak is generally expected for a red cell quantity about 3.0 mill. per c.mm. Simultaneously, the anemic state improved and the icteric index began to decrease. On the sixteenth day, the peripheral blood status was well within the normal range and choline therapy was discontinued. The icteric index at this time was 26 units. Marrow

was aspirated from the distal end of the body of the sternum at the level of the second interspace. The obtained specimen was worked up in the same manner as the first specimen. The gross marrow units were in size and number within the normal range. The hematocrit showed the various layers within the normal limits: fat 1; plasma 41; M-E 6; Er. 52 volumes per cent. The microscopic marrow units showed a remarkable adjustment toward normality and the fat spaces ranged between 20 to 35 per 25 Whipple ocular micrometer squares (Fig. 2-A).

We were not able to get simultaneous information on the status of the liver when choline therapy was discontinued because the patient refused to submit to another liver biopsy. We realize that the lack of this information weakens any conclusions we wish to draw with respect to the effect of choline on the liver fat; we nevertheless believe that a favorable action on the fat deposit had occurred because from the histologic point of view the liver was practically normal when the patient expired some months later from pneumonia (Figure 2-B).

DISCUSSION

For as yet unknown reasons, man may develop a spontaneous choline deficiency resulting in the production of a fatty liver and bone marrow organ. In certain individuals afflicted with Addison-Biermer's disease (Addisonian pernicious anemia), a progressive anemic state may develop as a sequel of the fatty metamorphosis. The latter state may well be enhanced by purified liver extracts because they contain no choline or amounts too small to be effective with respect to fat transportation and deposition. One author believes that the body stores of choline cannot be utilized to prevent development of a choline deficiency. This is of interest because our patient consumed 3 balanced meals a day and seemingly failed to utilize choline considered (13) to be a structural constituent of foods.

While we had no direct evidence of choline deficiency, it appeared to be most likely that the substance was deficient because of the conspicuous fatty liver and fatty bone marrow organ. The dramatic response of the anemic state and adjustment of the mean corpuscular diameter of the erythrocytes to choline therapy without supplementary antianemic substances would indicate that an adequate amount of hematopoietin was stored, although not elaborated by the fatty liver in sufficient amounts to prevent a deficiency state of the substance. Further, it is likely that fat deposition above a certain volume percentage in

³ If the recommended optical combination setup cannot be made, one may determine the normal microscopic marrow unit with any eyepiece and low power object available. Serial sections of an average normal gross marrow unit are moved under the Whipple micrometer and the number of squares noted that form the boundary of the maximum section.

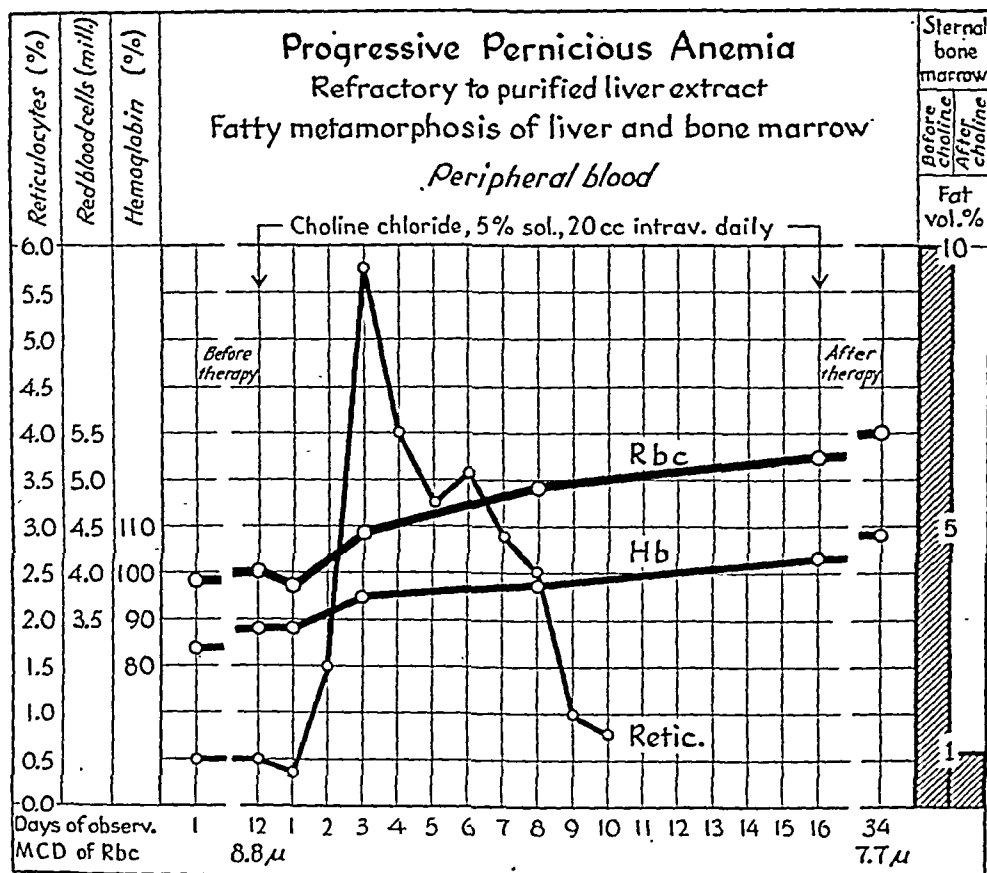


FIG. 3. PERIPHERAL BLOOD STATUS BEFORE, DURING, AND AFTER INTRAVENOUS CHOLINE CHLORIDE (MERCK) AND VOLUME PERCENTAGE OF STERNAL MARROW FAT, BEFORE AND AFTER CHOLINE THERAPY

the marrow organ may cause a disturbance in the utilization of the hematogenic substance should some be elaborated by the liver. While we were able to obtain data on the status of the bone marrow organ before and after choline therapy and on the liver before the administration of the lipotropic factor, we failed to get the cooperation of the patient for another liver biopsy when choline therapy was discontinued. As already mentioned elsewhere in this paper, the liver was found macroscopically and microscopically normal when the patient expired some months later from pneumonia. With the exception of the first dose, no ill effects nor any change of the blood pressure was observed during the administration of choline over the treatment period. Because of our inability to desensitize the patient to liver extracts, readministration of crude liver extracts could not be carried out. He was placed on Ventriculin

after the thirty-fourth day of observation. We could show in a clear-cut manner that an adequate amount of hematopoietin was stored in the liver and that the administration of choline in some way expedited the elaboration of the substance, bringing about a remission of the anemic state. The response to choline was in every respect compatible with that induced by potent liver extracts. Whether this type of therapy is effective in cases other than those afflicted with Addison-Biermer's disease (Addisonian pernicious anemia) must await further investigation. We see no reason why choline should not be supplemented when purified liver extracts are used, especially when a progressive anemic state develops and fatty metamorphosis of the liver and bone marrow organ appears to be the complicating factor. The remission of the anemic state on choline therapy is not in accord with the observation of one

worker (15) who gave choline hydrochloride to dogs made polycythemic and noted a depressing action of the substance on the erythropoiesis. It is worthwhile to note that others (1) employed blood transfusions to overcome the refractory anemic state.

SUMMARY

1. Report is made of a case afflicted with Addison-Biermer's disease (Addisonian pernicious anemia) who developed a progressive anemic state under adequate amounts of purified liver extracts.

2. The anemic state responded well to choline chloride (Merck), 5 per cent solution, 20 ml. intravenously for 16 days.

3. Biopsied liver and bone marrow tissue showed marked fatty metamorphosis of both organs. This fatty state was believed to have been the reason for the failure of the patient to maintain a normal blood status.

4. The fact that a remission of the anemic state, reduction of the mean corpuscular diameter of the erythrocytes, and return of the bone marrow organ toward a normal histology and volumetry occurred on choline therapy only seems to indicate that an adequate amount of hematopoietin (anti-pernicious anemia factor) was stored in the liver but not effectively elaborated because of the fatty state.

5. It is assumed that purified liver extracts contain either no choline or amounts too small to be effective with respect to fat transportation and deposition in certain individuals.

6. The amount of choline and the number of days of administration essential for the re-establishment of a physiologic state of the liver and bone marrow organ and elaboration of the hematopoietin in man await further investigation.

7. It is suggested that choline chloride (Merck) or substances containing choline may be supplemented when fatty metamorphosis of liver or bone marrow organ or both are present, particularly when a progressive anemia develops under ade-

quate purified liver extract therapy in the absence of factors known to interfere with a therapeutic response.

We wish to thank Dr. G. E. Fahr, Head of the Department of Internal Medicine, for his interest in the problem and for the permission to publish this case.

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THE CONVERSION OF HEMATIN TO BILIRUBIN FOLLOWING INTRAVENOUS ADMINISTRATION IN HUMAN SUBJECTS¹

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The results of earlier studies of the fate of hematin in the animal organism have been contradictory (1). Some have found that hematin, as well as hemoglobin, was converted quantitatively into bilirubin in bile fistula dogs (2). Partly as a result of this work and possibly also because of the ease with which hemoglobin is converted to hematin *in vitro*, it became rather generally accepted that hematin was intermediate in the pathway from hemoglobin to bilirubin. The studies of others (3 to 7) have indicated, however, that the transition is over a "green" hemoglobin, or biliverdin-iron-globin, rather than over hematin. Thus the present question is not so much whether hematin is a normal precursor of bilirubin, but whether, once formed under abnormal conditions, it is capable of conversion to bilirubin *in vivo*. This question is of considerable fundamental significance because of the presence of hematin in the circulating blood in a variety of pathological states such as gas bacillus sepsis, pernicious anemia, hepatic disease (8, 9), severe malaria, and black-water fever (8, 10). As Fairley (10) has shown, hematin in the circulating blood is bound to the serum albumin, a compound which he has designated as "methemalbumen." The fate of hematin is also of considerable interest because of its probable identity with the so called "malarial pigment" (11).

Certain investigators in recent years have dissented from the view that hematin is converted to bile pigment. Bingold (12) and Duesberg (9), in particular, have designated hematin as a "blind alley" in hemoglobin metabolism. The literature pertaining to this question was reviewed in 1938 (1) so that this need not be given in more

detail at present. It is desirable, however, to refer in some detail to Duesberg's studies (9) since these have provided the most concrete experimental data favoring Bingold's concept. Duesberg (9a) found that hemoglobin was promptly converted to bilirubin when injected either into the circulating blood or into ascitic fluid within the peritoneal cavity. On the contrary, no evidence of conversion of hematin to bilirubin was noted under like conditions. To anticipate the criticism that the injected hematin might have been converted to bilirubin, and then at once excreted by the liver, Duesberg used subjects with complete biliary obstruction, reasoning that if there were conversion in such individuals the bilirubin level should increase. Actually, increases were noted after hemoglobin injection, but not after hematin. In other experiments in non-jaundiced individuals, Duesberg did not find any increase of bilirubin in the bile following intravenous injections of hematin. Obviously, it was not possible to obtain data over long periods of time nor to control the factor of varying dilution of the bile in these studies. The determination of urobilinogen excretion in the feces would have been preferable, but this was not followed. So far as can be determined, Gitter and Heilmeyer (13) are the only investigators who injected hemoglobin and hematin and then determined the amount of urobilinogen in the feces. Gitter and Heilmeyer found that hemoglobin injection was regularly followed by increased urobilinogen excretion, quite in accord with previous observations. Their data for the hematin experiments, however, were inconclusive. For the most part, a definite increase of urobilinogen was not observed.

It may be noted that Duesberg and Hagenbeck (14) observed the appearance of hematin in the plasma after intravenous injection of hemoglobin in subjects with liver disease, especially severe cirrhosis of the liver. They regarded this, in fact,

¹ Aided by a grant from the Medical Research Fund, Graduate School, University of Minnesota.

² Dr. Pass was killed in an automobile accident in 1939. At that time, he had completed investigations which were to have been compiled as a thesis for the Ph.D. degree in Medicine. The present report includes a part of these studies.

as a means of testing liver function, since hematin did not appear in the normal subjects receiving hemoglobin intravenously.

The purpose of the present investigation was to observe the serum bilirubin after injections of hemoglobin and hematin, in subjects without evidence of liver or biliary tract disease and in patients with complete biliary obstruction, and to determine the urobilinogen excretion in the feces of the former group in order to ascertain whether hemoglobin and hematin injections are followed by equal increase or whether hemoglobin alone is converted to bile pigment.

MATERIAL AND METHODS

Crystalline hemin was prepared from washed human red blood cells according to a modification of Schälfejeff's method (15). The crystals as obtained from glacial acetic acid were washed repeatedly with water and were recrystallized from pyridine chloroform (15). The solutions of hematin injected intravenously were prepared by dissolving the crystalline hemin in 10 ml. of 5 per cent sodium carbonate which had just been brought

to the boiling point. The solution was then diluted to 50 ml. with sterile distilled water, making a concentration of approximately 1 per cent carbonate. The amount of hematin dissolved varied between 100 and 750 mgm. as noted in Figures 1 to 12. The oxyhemoglobin solutions which were used were prepared in each instance from the blood of the individual under investigation. The red blood cells were washed repeatedly with sterile physiological saline and were then laked with sterile distilled water. Under sterile conditions the hemolyzed blood was then centrifuged at 3000 R.P.M. for one half hour after which the supernatant hemoglobin solution was carefully taken directly into a large sterile syringe from which it could be given intravenously. A small measured portion was removed for hemoglobin determination, which was carried out with a Zeiss stufenphotometer, according to Heilmeyer's method (16).

Hematin was administered intravenously in 11 subjects. Four of these were normal individuals, 1 suffered from spontaneous hypoglycemia, and 1 from chronic glomerulonephritis (without uremia). Four had complete biliary obstruction due to cancer of the extrahepatic biliary tract. One had a complete external biliary fistula as a result of a secondary operation for benign (postoperative) common duct stricture. In separate experiments, oxyhemoglobin was also administered

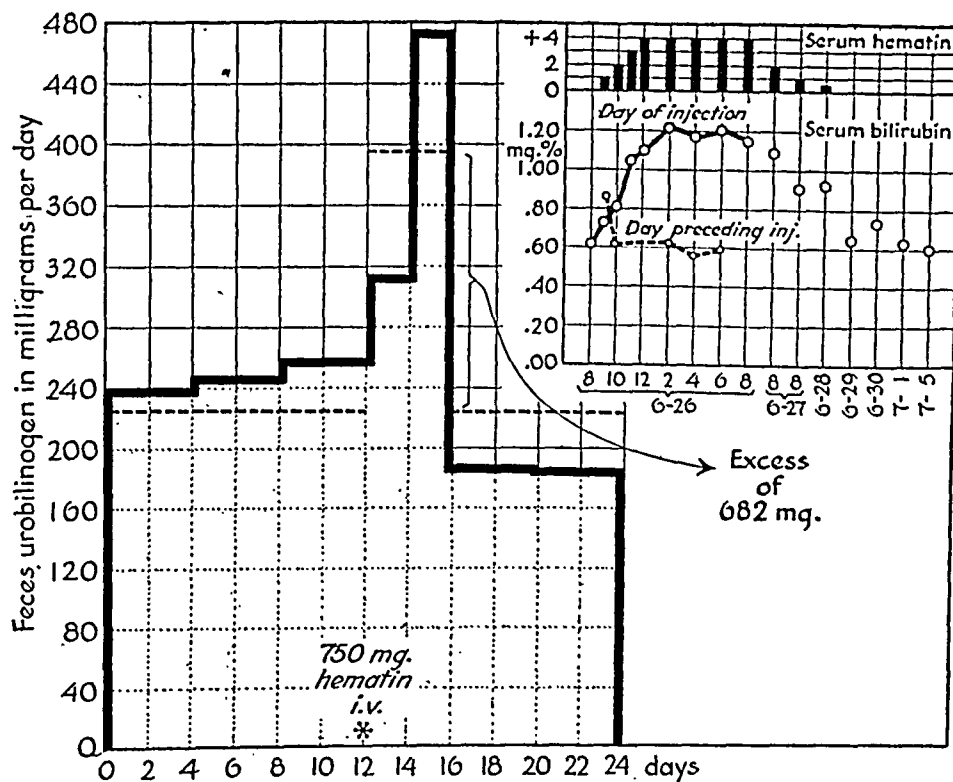


FIG. 1. INTRAVENOUS INJECTION OF HEMATIN IN A NORMAL MALE SUBJECT, W. Z., AGE 24

The increase of the feces urobilinogen roughly corresponds in amount with the injected hematin. The higher levels of serum bilirubin and the long persistence of hematin in the serum, after the injection, may be noted.

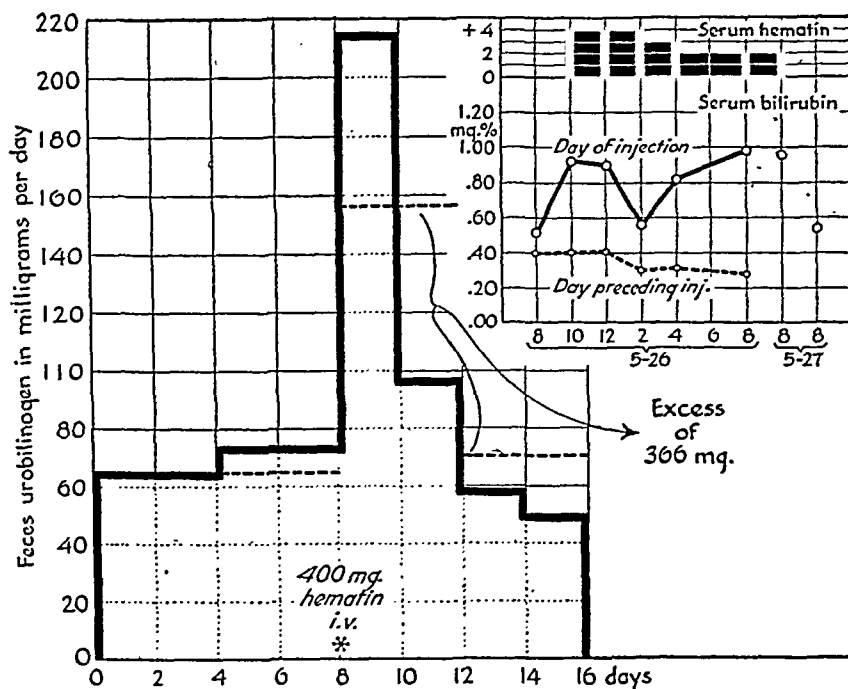


FIG. 2. INTRAVENOUS INJECTION OF HEMATIN IN A NORMAL FEMALE SUBJECT, E. S., AGE 21

The increase of the feces urobilinogen, roughly corresponding in amount to the injected hematin may be noted, together with the higher levels of serum bilirubin and long persistence of hematin in the serum.

intravenously to 3 of these subjects, including the patient with hypoglycemia, 1 of the normal individuals, and 1 of the cases of complete biliary obstruction.

Bilirubin in serum and urine was determined with the stufenphotometer by a modification (17) of the Jendrassik-Czike technique (18).

Hematin in the serum was followed by means of conversion to hemochromogen, effected by addition of a few drops of ammonium sulphide, the characteristic hemochromogen absorption band at $557\text{ m}\mu$ being observed in a Zeiss grating spectrometer. No attempt was made to quantitate the hemochromogen absorption in these studies. The relative intensity of the band was simply indicated as either 0, trace, 1+, 2+, 3+, or 4+, employing in each instance a test tube of the same diameter in which to inspect the absorption spectrum of the solution, in accordance with Schumm's method (8).

Urobilinogen in the feces and urine was determined by means of Watson's modification (19) of Terwen's method (20).

RESULTS

The results are shown in Figures 1 to 12, inclusive. They indicate at least partial conversion of hematin to bile pigment. The data for the fecal urobilinogen excretion following hematin ad-

ministration constitute the clearest evidence of conversion. The augmentation of the fecal urobilinogen after hematin injections was just as quantitative as after injection of comparable amounts of hemoglobin (in terms of the pigment fraction). This is seen in Figures 1 to 5, inclusive. In at least 4 out of 5 experiments (Figures 1, 2, 4, 5), the excess of urobilinogen excreted corresponded roughly with the amount of hematin injected. In 1 of the 5 experiments of this group (Figure 3) the findings are somewhat more difficult of interpretation. If the excess in this instance is calculated for the 4-day period after hematin injection, as was done in the other 4 experiments, then it amounts to but roughly 30 per cent of the amount injected. The excess for the first 2 days, however, was slightly more than 60 per cent of what might have been expected if all the hematin were excreted as urobilinogen. This brings up another question, i.e., whether an excess of pigment injected into, or formed in, an otherwise normal individual may be expected to reappear quantitatively as urobilinogen. The

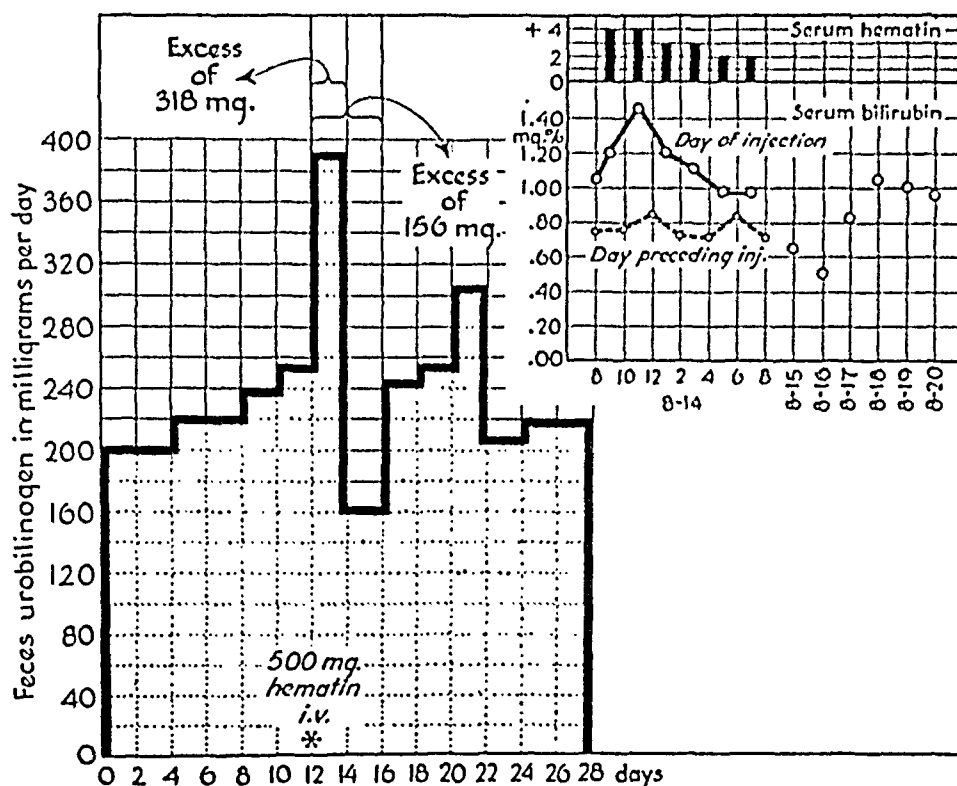


FIG. 3. INTRAVENOUS INJECTION OF HEMATIN IN A NORMAL MALE SUBJECT, S. S., AGE 20

The increase of the feces urobilinogen is from 30 to 60 per cent of the injected hematin, depending on the period for which the excess following injection is calculated. The levels of serum bilirubin are distinctly higher than on the days preceding or after the injection.

majority of the evidence on this point is in the affirmative (1, 21). While the calculation of the excess urobilinogen for a 4-day period, following hematin or hemoglobin injection, is perhaps open to question, the data shown in Figures 1 to 5, inclusive, leave no doubt that the hematin gave rise to considerable amounts of bile pigment, as represented by the increased urobilinogen excretion. The data for the serum bilirubin following hematin injections, as shown in Figures 1 to 3, inclusive, and in Figure 6, is quite in accord with the belief that the hematin was converted to bilirubin. Figure 6 is of particular interest in revealing the different manner in which hemoglobin is handled, as compared with hematin, injection of the former resulting in a prompt sharp rise and fall of the serum bilirubin, while after hematin the increase was much smaller but more prolonged.

The observations in the patient with the total external biliary fistula (Figure 7) are very difficult of interpretation, principally because of the marked spontaneous fluctuation in bilirubin excretion in the bile.

The principal criticism of this experiment is that the amounts of hematin injected were too small in the face of the fluctuant native bilirubin excretion just mentioned. The question arises as to whether any excess of bilirubin, derived from hematin, continues to be excreted for longer than 24 hours. If the excess is calculated for the first 24 hours only, then it is seen that the first injection of 100 mgm. was followed by an excess of 87 mgm. After the second injection of 200 mgm., however, the excess was but 35 mgm., very much less than might have been expected. Another disturbing fact in this experiment was the high level of 212 mgm. noted several days later, quite spontaneously. Thus it is evident that while the data in Figure 7 suggest some conversion of hematin to bilirubin, they are not conclusive.

The studies of the serum bilirubin after injection of hematin or hemoglobin, in jaundiced subjects, has provided somewhat conflicting data, as seen in Figures 8 to 12, inclusive. The evidence

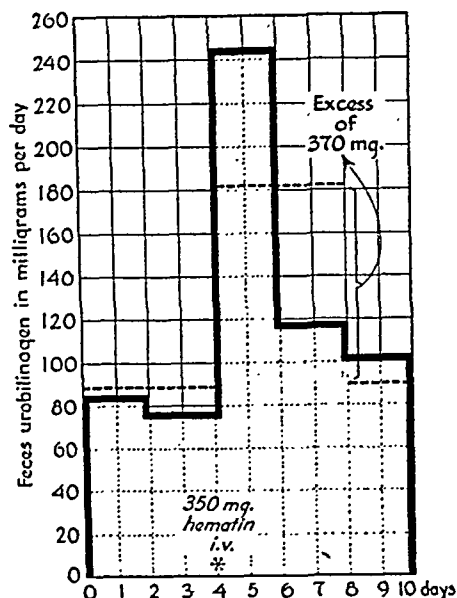


FIG. 4. INTRAVENOUS INJECTION OF HEMATIN IN A 28-YEAR-OLD FEMALE SUBJECT, L. H., WITH QUIESCENT CHOLECYSTITIS AND CHOLELITHIASIS

The excess of the feces urobilinogen after the injection corresponds roughly with the amount injected.

in these experiments for conversion of hematin to bilirubin in the circulating blood is not uniformly convincing. The most carefully conducted ex-

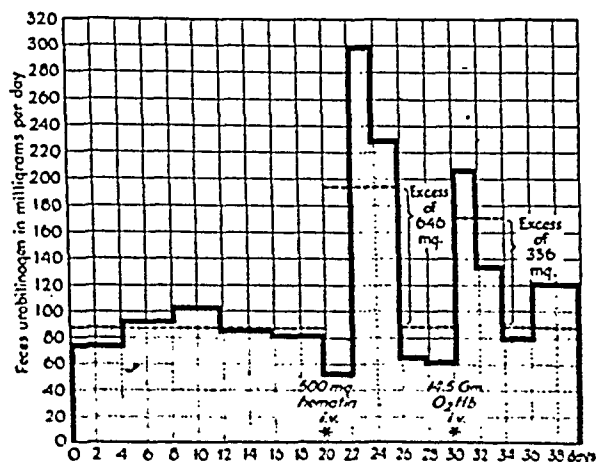


FIG. 5. INTRAVENOUS INJECTION OF HEMATIN AND OF OXYHEMOGLOBIN, IN SEPARATE EXPERIMENTS, IN A MALE PATIENT, AGED 60, HOSPITALIZED BECAUSE OF SPONTANEOUS HYPOGLYCEMIA (ADENOMA OF ISLANDS OF LANGERHANS)

The excess of the feces urobilinogen after the hematin injection is somewhat greater, and that after hemoglobin is smaller, than expected. In spite of this, it is clear that bile pigment was produced in both instances.

periment of this group is shown in Figure 12, where it is seen that significant rises in serum bilirubin occurred after both hematin and hemoglobin, but again in somewhat more marked fashion after hemoglobin. Following each of these increases, a distinct augmentation in the urine bilirubin is observed. Whether this is significant cannot be determined with certainty, although the time sequence suggests that it is. An appreciable rise in serum bilirubin, following the hematin injection, is noted in Figures 8 and 11, but, in the former, the value is noted to be just as high on

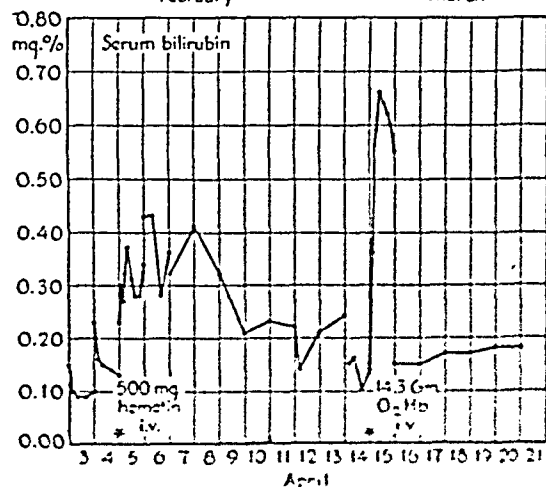
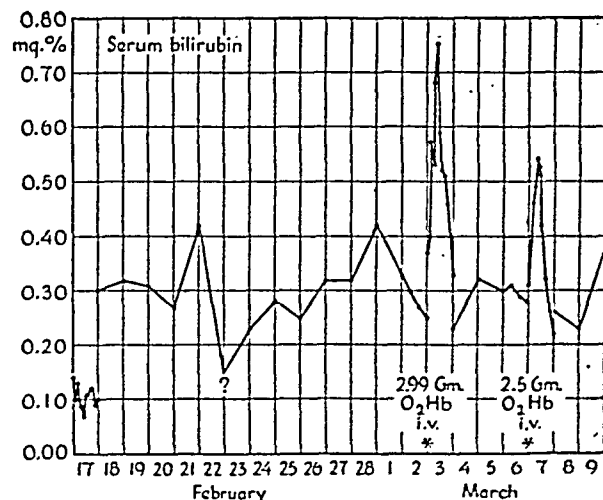


FIG. 6. SERIES OF EXPERIMENTS IN A MALE PATIENT, S. J., AGE 67, WITH SPONTANEOUS HYPOGLYCEMIA (SAME INDIVIDUAL AS IN FIG. 5)

The sharp increases of the serum bilirubin after each injection of oxyhemoglobin, as compared with the faster and less convincing rise in the curve after hematin, may be contrasted.

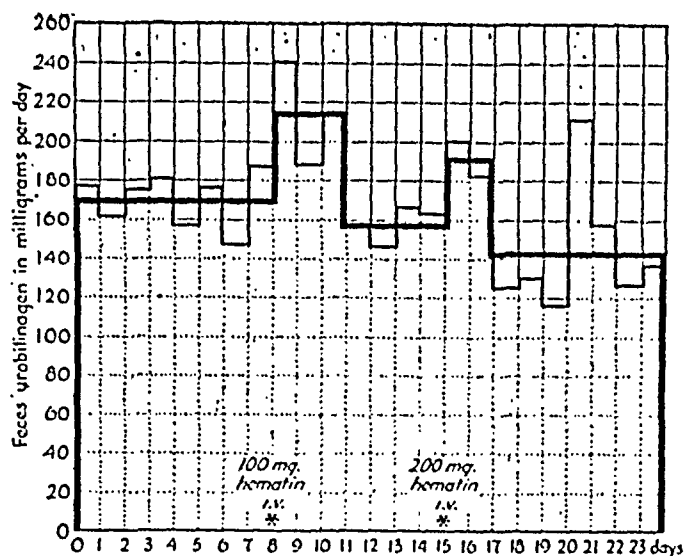


FIG. 7. INTRAVENOUS INJECTIONS OF HEMATIN IN A FEMALE SUBJECT, M. L., AGE 52, HAVING A TOTAL EXTERNAL BILIARY FISTULA

The heavy black line is the average for the entire period indicated. It is evident that the calculation of excesses is somewhat arbitrary, also that the excess after 200 mgm. was smaller than after 100 mgm. and that a marked spontaneous increase occurred a number of days later. The results here are regarded as of questionable significance.

the next day when hematin was not given. In the latter experiment (Figure 11), the rise in serum bilirubin was not reproducible with a second hematin injection a week later, although it may be noted that on the first occasion the rise was observed only 2 hours after the injection while on the subsequent occasion the serum was not examined until 4 hours afterwards. A similar criticism may be made of the other experiments of this group. No increase was observed in the experiment shown in Figure 9, again at 4-hour intervals after injection. In Figure 10, a distinct rise is seen, but not until 12 hours after the injection, so that it is of questionable significance.

DISCUSSION

The marked augmentation of urobilinogen in the feces following hematin injection in non-jaundiced subjects leaves no doubt that the hematin was converted to bilirubin and thence, after excretion in the bile and reduction in the intestinal tract, to urobilinogen. The evidence for rapid conversion of hematin to bilirubin in the circulating blood is not as clear. Sharp rises in serum bilirubin were noted after hemoglobin in-

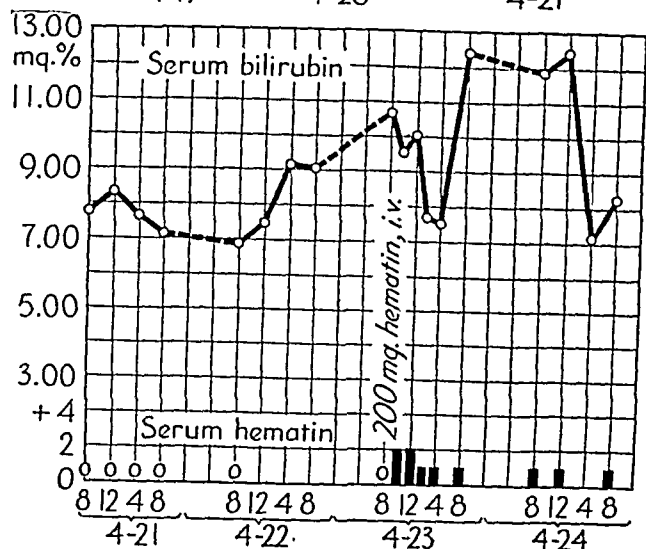
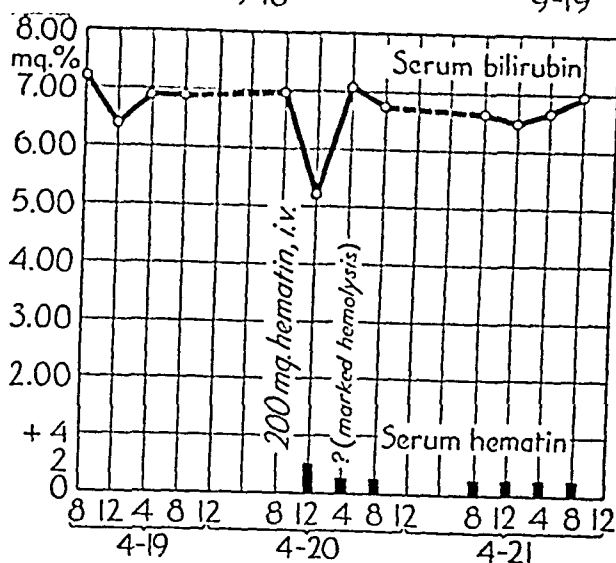
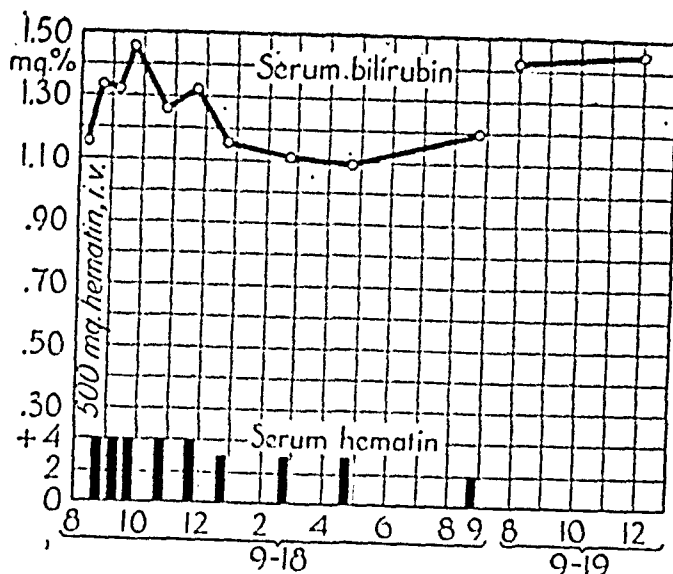


FIG. 8. INTRAVENOUS INJECTION OF HEMATIN IN A MALE SUBJECT, AGE 58, HOSPITALIZED BECAUSE OF CHRONIC GLOMERULONEPHRITIS

An apparent elevation of serum bilirubin is seen following the hematin, but on the next day the values were just as high without hematin. It may be noted, however,

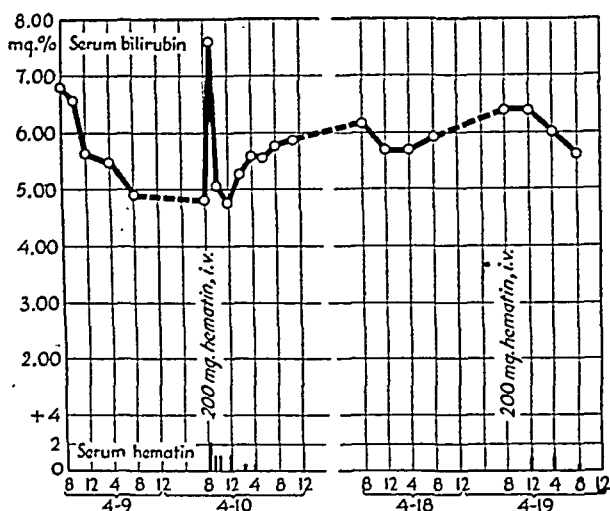


FIG. 11. INTRAVENOUS INJECTIONS OF HEMATIN IN A MALE SUBJECT, M. L., AGE 52, HOSPITALIZED BECAUSE OF COMPLETE BILIARY OBSTRUCTION DUE TO CANCER OF THE COMMON BILE DUCT

After the first injection, a brief transitory increase of serum bilirubin, of doubtful significance, is seen. Nothing of significance was observed in the second experiment.

jections, while the increase following hematin was not as uniform, nor when it did occur, as convincing, with one exception (Figure 12). The difference is not due to amount of available pigment introduced, since, on one occasion (Figure 6), a characteristic sharp rise is observed after but 2.5 grams of hemoglobin, equivalent to 100 mgm. of hematin; while on a different occasion, 500 mgm. of hematin produced only a slow and somewhat prolonged increase which might even be interpreted as nothing more than a cyclic variation. The data for serum bilirubin following hematin injection

that hematin was present in the serum at least until 9 p.m. on the 18th.

FIG. 9. INTRAVENOUS INJECTION OF HEMATIN IN A FEMALE SUBJECT, B. E., AGE 68, HOSPITALIZED BECAUSE OF COMPLETE BILIARY OBSTRUCTION DUE TO CANCER OF THE PANCREAS

In this instance, a temporary drop in serum bilirubin is seen following the hematin. The long persistence of the latter in the serum is noted.

FIG. 10. INTRAVENOUS INJECTION OF HEMATIN IN A MALE SUBJECT, W. B., AGE 69, HOSPITALIZED BECAUSE OF COMPLETE BILIARY OBSTRUCTION DUE TO CANCER OF THE PANCREAS

A transitory decline followed by a considerable increase in serum bilirubin is seen, following the hematin. Persistence of hematin in the serum into the second 24 hours is noted.

tions, as shown in Figures 1, 2, 3, 6, 11, and 12, may be accepted as supporting a conversion of hematin to bilirubin. That noted in Figures 8, 9, and 10 is equivocal and might readily enough be interpreted as supporting the conclusions of Bingold and Duesberg, which have already been mentioned. These data do not exclude, however, a removal of hematin by the liver with subsequent conversion to bilirubin and biliary excretion as first suggested by Aschoff (22). In the subjects with obstructive jaundice, biliary excretion would not be expected, and, as Duesberg pointed out, a conversion of hematin to bilirubin ought to be followed here by an elevation of the serum bilirubin. This assumes, however, that any excess formed in this way is not rapidly excreted in the urine, a possible source of loss which has not hitherto been taken into account. In the 2 experiments shown in Figure 12, 1 with hematin and 1 with hemoglobin, distinct increases in serum bilirubin are seen which are followed by significant increases in urinary bilirubin. The increase of the serum bilirubin after hematin, although distinct, was not as striking as that after hemoglobin,

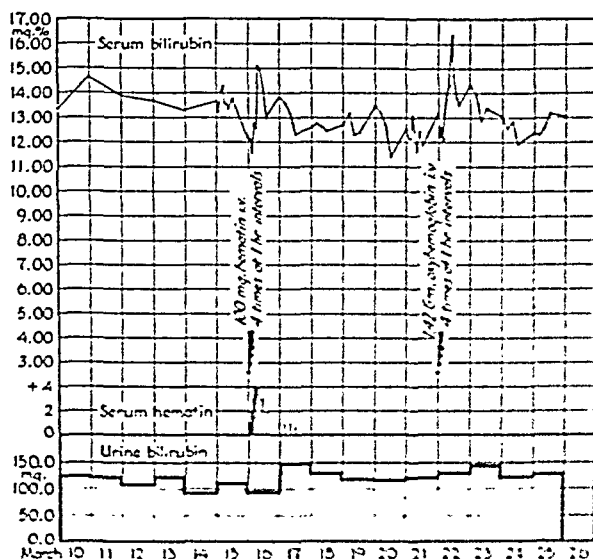


FIG. 12. REPEATED INTRAVENOUS INJECTION OF HEMATIN AND OF HEMOGLOBIN IN SEPARATE EXPERIMENTS IN A MALE SUBJECT, J. H., AGE 55, HOSPITALIZED BECAUSE OF COMPLETE BILIARY OBSTRUCTION DUE TO CANCER OF THE PANCREAS

Distinct increases of the serum bilirubin are observed after injection of each of the two substances. Subsequent increases of the urine bilirubin are likewise noted.

again in spite of the fact that the amount of hematin injected was about 40 per cent larger. The results of this experiment are regarded as the most significant of any in this group. It is quite possible that the lack of elevation of serum bilirubin following hematin in other experiments (Figures 9 and 10) was due in part to an increased excretion (of bilirubin) in the urine. Another factor may be that of a very slow conversion of hematin to bilirubin as contrasted with a more rapid one for hemoglobin. With respect to the latter possibility, the long persistence (20 to 36 hours) of hematin in the serum, after injection, indicates a slow rate either of conversion or of removal. This may very well be related to the formation of Fairley's methemalbumen (10). Unfortunately, data were not obtained in the present studies on the disappearance rate of hemoglobin, so that direct comparisons are impossible. It is well known, however, that hemoglobin disappears from the circulation relatively rapidly even if concentrations below the renal threshold are considered. Furthermore, the rapid increases of serum bilirubin after injection of hemoglobin, as noted particularly in Figure 6, indicate the speed with which hemoglobin might be expected to disappear.

A very slow conversion of hematin to bilirubin, either in the blood or the liver, would adequately explain Duesberg's failure to note any increase in bilirubin in duodenal drainage samples obtained over relatively short periods.

SUMMARY AND CONCLUSIONS

1. Following intravenous administration of hematin in non-jaundiced human subjects, an augmentation of the feces urobilinogen was observed which was roughly proportional to the amount of hematin given. This is interpreted as evidence of formation of bile pigment from hematin, contrary to the views of Bingold and Duesberg who believe that hematin is not converted to bilirubin.

2. The serum bilirubin was not uniformly elevated following the hematin injections, in contrast with those of hemoglobin which regularly produced sharp rises. Nevertheless, significant elevations were noted after hematin in a number of experiments. A relatively slow conversion of

hematin to bilirubin is suggested by the long persistence of hematin in the circulating blood. This may be related to formation of methemalbumen. Increased excretion of bilirubin in the urine may account in part for failure of the serum bilirubin to rise after hematin injections in subjects with complete biliary obstruction.

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MECHANISM OF THE POSITIVE CEPHALIN-CHOLESTEROL FLOCCULATION REACTION IN HEPATITIS

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An alteration of the blood serum proteins in patients with catarrhal jaundice has been amply established by the development of a positive cephalin flocculation (1). A standard emulsion composed of cephalin and cholesterol, which remains stable in 0.2 ml. to 0.1 ml. normal serum, diluted 1:21, tends to flocculate and precipitate when added under identical conditions to the serum of patients with catarrhal jaundice. As the disease subsides, the positive reaction returns to negative. The mechanism of the reaction has not been adequately explained.

We have shown in unpublished experiments that a positive reaction is accompanied by the absorption of globulin by the emulsion particles, since rabbits immunized intravenously with washed flocculated material develop precipitins for the globulin components of both the euglobulin and pseudoglobulin fractions of human serum. The intensity of the precipitin reaction is the same with normal serum and with that from hepatitis, indicating that the absorbed protein does not differ antigenically from the globulin of normal serum.

The mechanism of the cephalin-cholesterol flocculation reaction has been investigated (2) and it was found that the gamma globulin fraction of the serum was the sole component giving a positive flocculation, and further that there was no difference in the flocculating power of the gamma globulin fraction obtained electrophoretically from a normal serum giving a negative reaction and the gamma globulin obtained from a hepatitis serum giving a strongly positive reaction. It was inferred that the negative flocculation obtained with normal sera was due to the inhibiting action by some other component of the serum. Complete inhibition of the reaction was not obtained with the albumin fraction in the proportions used by these observers, although inhibition of the colloidal gold reaction, which showed many points of similarity,

was obtained with electrophoretically separated albumin. The present report is an extension of the studies mentioned above, including the use of gamma globulin and albumin in amounts comparable to those occurring in 0.1 ml. of whole serum.

EXPERIMENTAL

Serum was taken from a number of normal human subjects giving a negative flocculation reaction and from 2 patients with obvious liver disease giving strongly positive (++++) flocculation reactions. Patient 1 was suffering from acute fulminating post-arsphenamine hepatitis. Patient 2 was in the first week of catarrhal jaundice. Electrophoretic analysis and separation of the sera were carried out in the Tiselius apparatus, using a buffer consisting of 0.02 *M* sodium phosphate + 0.15 *M* NaCl, at pH 7.4, and the concentration of each fraction of albumin and gamma globulin obtained was determined by measuring its refractivity in a previously calibrated Zeiss interferometer.

Cephalin flocculation tests were performed on these separated serum fractions, in the manner previously described (3), using diminishing amounts of the gamma globulin fraction alone, and also after the addition of various albumin fractions. The protein preparations were mixed with 4 ml. of merthiolated saline and 1 ml. of standard cephalin-cholesterol emulsion. The tubes were allowed to stand at room temperature for 48 hours. Readings were made at 24 and 48 hours and recorded in terms of negative to ++++.

RESULTS

The effect of increasing dilution of the isolated gamma globulin fractions on cephalin-cholesterol flocculating properties

The observations of others (2) on the cephalin-cholesterol flocculating properties of gamma globulin were confirmed and extended by adding decreasing amounts of the isolated gamma globulin preparations, from various sources, to a series of tubes containing 4 ml. saline and standard cephalin-cholesterol emulsion, to determine the degree of

TABLE I

Cephalin flocculation shown by diminishing amounts of the isolated gamma globulin fraction of normal sera and of sera of hepatitis cases

	Gamma globulin fraction in diminishing amounts				
	0.55 to 0.65 mgm.	0.44 to 0.52 mgm.	0.33 to 0.39 mgm.	0.22 to 0.26 mgm.	0.11 to 0.13 mgm.
Normal case I	+++++	+++++	+++++	+++	±
Normal case II	QNS	+++++	+++++	+++	0
Normal case III	+++++	+++++	+++++	±	0
Normal case IV	+++++	+++++	+++++	±	0
Hepatitis case I	+++++	+++++	+++++	+++	0
Hepatitis case II	+++++	+++++	+++++	+++	0

dilution necessary to obliterate the flocculation (Table I).

It will be noted that as the gamma globulin fractions are progressively diluted, the flocculation diminishes, and is usually abolished at a concentration between 0.22 and 0.11 mgm. of gamma globulin in 5.2 ml. of diluent. No significant differences in flocculating properties were noted with the gamma globulin fraction from hepatitis sera when compared with a similar fraction from normal serum. In no instance, under similar conditions, did an albumin fraction give a positive flocculation.

It is noteworthy that the protein fractions used in these studies were separated electrophoretically in a phosphate buffer at pH 7.4 near the isoelectric range (pH 6 to 7) of gamma globulin. The gamma globulin fraction obtained at a pH of 8.5, using a barbital buffer, failed to show flocculating properties, even after dialyzing to restore the pH to 7.4.

The effect of albumin from normal serum and from the serum of hepatitis on the flocculating properties of gamma globulin

A series of tubes was prepared containing diminishing amounts of isolated gamma globulin similar to those described above, and to each was added 5.9 mgm. (or 5.4 mgm.) of electrophoretically separated albumin (the approximate amount calculated to be present in 0.1 ml. of the normal serum used). Cephalin-cholesterol emulsion was then added in the usual fashion and the degree of flocculation recorded after 48 hours (Table II).

It will be noted that in the proportions used, considerable inhibition of the flocculating properties of the gamma globulin fraction is obtained with an albumin fraction from normal sources. In contrast, an equal amount of the albumin fraction from the 2 cases of hepatitis showed distinctly less inhibiting power on the flocculating properties of gamma globulin.

DISCUSSION

The elaboration of the serum albumins is usually ascribed to the liver. When this organ becomes diseased, such as in long-standing cirrhosis, a distinct drop in the serum albumin fraction is common, as well as an increase of serum globulin, especially of the gamma globulin fraction. It has recently been pointed out (4) that in acute hepatitis also, while similar changes are not striking by the usual salting-out methods (5), diminution of albumin and increase of gamma globulin are demonstrable by electrophoretic analysis. The studies

TABLE II

Comparison of inhibiting powers of albumin fraction of normal serum and of albumin fractions from cases of hepatitis

	Gamma globulin fraction in diminishing amounts				
	0.55 mgm.	0.44 mgm.	0.33 mgm.	0.22 mgm.	0.11 mgm.
Gamma globulin fraction from hepatitis case I alone	++++	++++	++++	+++	0
Gamma globulin fraction from case I + 5.9 mgm. of albumin fraction from normal serum	+++	++	0	0	0
Gamma globulin fraction from case I + 5.9 mgm. of albumin fraction from hepatitis case I	++++	++++	+++	+++	QNS
	0.65 mgm.	0.52 mgm.	0.39 mgm.	0.26 mgm.	0.13 mgm.
Gamma globulin fraction from hepatitis case II alone	++++	++++	+++	++	0
Gamma globulin fraction from case II plus 5.4 mgm. of albumin fraction from normal serum	±	±	0	0	0
Gamma globulin fraction from case II plus 5.4 mgm. of albumin fraction from hepatitis case II	+++	+++	±	0	0

TABLE III
Protein fractionation

	Electrophoretic fractionation *							Salt fractionation (Howe method (5))			
	Total protein	Albumin	Globulin				A/G ratio	Total protein	Albumin	Globulin	A/G ratio
			α	β	γ	Total					
Case II	980	485 50 per cent	100 10 per cent	185 19 per cent	210 21 per cent	495 50 per cent	0.98	7.2	4.6	2.6	1.76
Normal	1055	665 63 per cent	80 8 per cent	195 18 per cent	115 11 per cent	390 37 per cent	1.70	7.3	5.4	1.9	2.84

* Expressed in arbitrary units and percentages derived from descending pattern areas.
Buffer 0.02 M sodium phosphate + 0.15 M NaCl; pH 7.4.

reported here confirm these observations (Table III). The data presented indicate that during the active stage of hepatitis an alteration occurs in the albumin fraction as reflected by the cephalin flocculation reaction. The chemical nature of this alteration is at present under investigation. Profound changes in serum albumins have been demonstrated in nephrosis by immunological studies (6), and by electrophoresis (7), but modification of the albumin fraction in active hepatitis has not been recognized previously.

It is recognized that the effect on cephalin flocculation here described may not be a function of true albumin, and, until further investigation, should be considered a function of the fraction separated under the conditions employed. Subfractions of albumin have already been made and it is well recognized that the electrophoretic fraction used in these experiments represents a mixture.

A hypothesis for the mechanism of the cephalin-cholesterol flocculation reaction can therefore be advanced. The gamma globulin component of normal serum fails to bring about flocculation because of an inhibitory action exerted by substances in the electrophoretically separated albumin fraction. In disease, a positive flocculation may be obtained with a serum due to any of the following alterations: (1) Increase of gamma globulin in such quantity that there is insufficiency of the normal components of the serum albumin fraction to inhibit the reaction; (2) diminution of the serum albumin fraction below initial levels

necessary to inhibit the reaction; (3) diminution in the flocculation-inhibiting properties of the albumin fraction, such as has been demonstrated above. The positive test observed in hepatitis etc., is probably due to a combination of all these factors, especially to modification of the albumin fraction.

CONCLUSIONS

(1) The flocculation of a cephalin-cholesterol emulsion is observed with the isolated gamma globulin fraction obtained from normal serum and from the serum of cases of hepatitis.

(2) The flocculating properties of gamma globulin are not affected in cases of hepatitis, although there may be an increase of this fraction in the serum during the course of the disease.

(3) The electrophoretically separated albumin fraction from normal sera in sufficient concentration tends to inhibit the flocculation of the gamma globulin fraction, whereas the albumin fractions from the sera of cases with hepatitis show relatively less inhibition of the flocculation.

(4) An hypothesis for the mechanism of the cephalin-cholesterol reaction is proposed.

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SIGNIFICANCE OF CEPHALIN-CHOLESTEROL FLOCCULATION TEST IN MALARIAL FEVER¹

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The effect of malarial fever on hepatic physiology has recently been investigated. It has been found (1) that considerable bromsulphthalein retention, a marked reduction in cholesterol and cholesterol esters, a moderate fall in phospholipids, diminished hippuric acid excretion and a strongly positive cephalin-cholesterol flocculation reaction resulted when malaria was induced in 9 patients by inoculation with *Plasmodium vivax* for the treatment of central nervous system syphilis. Other investigators (2) studied 10 patients with acquired malaria in all of whom the cephalin-cholesterol reaction was positive. The aforementioned authors (3) noted a hypoproteinemia with a decrease in serum albumin and an increase in globulin in patients with therapeutic malarial fever. In this communication, data are presented dealing with changes in the various serum protein fractions occurring during malarial fever, and correlating these findings with the cephalin-cholesterol flocculation test.

MATERIAL

Six patients suffering from central nervous system syphilis were infected with *Plasmodium vivax* by the intravenous injection of malarial blood. Prior to the institution of malarial therapy each patient was subjected to a number of liver function tests and blood studies, which included determination of the prothrombin time (4), bromsulphthalein retention, using 5 mgm. of dye per kgm. body weight with determinations 5 and 30 minutes after injection (5), alkaline phosphatase (6), serum bilirubin (7), serum cholesterol (8), cephalin-cholesterol flocculation (9), serum albumin and globulin by the salting-out method (10), occasional hippuric acid excretion tests; urine collected 1 hour after intravenous injection of 1.77 grams of sodium benzoate (11), non-protein nitrogen (12), uric acid (13), and electrophoretic analysis of the blood serum (14). These and other studies were repeated at intervals during the

course of the malarial fever and also during the period of antimalarial therapy and convalescence. The patients in this group experienced from 74 to 134 hours of fever, 103° F. or over.

RESULTS

All 6 patients developed a positive cephalin-cholesterol flocculation reaction during the course of malarial fever. In 4 patients, the test became positive after about 20 hours of fever, in 1 patient after 35 hours, and in the remaining patient after 70 hours. The test finally became 4 plus in 5 of 6 cases and 3 plus in the remaining case after 80 hours of fever. There was a marked reduction in serum cholesterol in all cases, some bromsulphthalein retention in 3 of the 6, and an elevated serum bilirubin in 1 instance. Definite hypoproteinemia and hypoalbuminemia occurred in all cases. A slight increase in globulin was noted in 5 cases of the 6 (Table I), using the Howe method. No consistent significant changes were found in prothrombin time or in alkaline phosphatase values. Hippuric acid excretion studies were performed in only 2 cases and 1 showed no evidence of diminished excretion. Observations on a typical case are shown in Table II.

Electrophoretic analysis and separation of albumin and gamma globulin fraction were performed using the Tiselius method (14) with a number of sera as outlined in the preceding paper (15). Fractions were obtained from sera taken both before the institution of malarial therapy and before its termination. Electrophoretic separations were carried out in a buffer composed of 0.02 M sodium phosphate and 0.15 M sodium chloride at pH 7.4 and the concentration of each fraction of albumin and gamma globulin obtained was determined by measuring its refractivity in a previously calibrated Zeiss interferometer (Figure 1).

¹ Aided in part by a grant from the William J. Matheson Commission.

TABLE I

Showing the maximum change of some constituents of blood serum in patients with Plasmodium vivax infections

Case no.	No. of hours with T. of 103° or over	Weight loss	Cephalin flocculation		Serum bilirubin*	Total protein†		Albumin†		Globulin†		Cholesterol		Bromsulphthalein‡
			Before	During fever		Before	During fever	Before	During fever	Before	During fever	Before	During fever	
		lbs.			mgm. per cent	grams per 100 ml. serum						mgm. per cent		per cent
1	74	8	0	4+	0.9	7.0	5.7	5.0	3.3	2.0	2.4	205	65	80/18
2	88	6	0	4+	1.8	7.5	5.2	5.0	2.9	2.5	2.3	292	114	95/35
3	79	4½	0	4+	0.5	7.1	5.8	5.1	3.2	2.0	2.6	196	92	75/5
4	134	?	0	4+	0.5	6.4	5.8	4.3	2.9	2.1	2.9	231	82	55/5
5	87	7	0	4+	0.5	7.5	6.7	5.2	3.9	2.3	2.3	242	133	80/0
6	80	6	0	3+	0.5	6.2	5.8	4.3	3.6	1.9	2.2	322	149	70/10

* Highest value.

† By the salting-out method of Howe.

‡ Maximum retention using 5 mgm. of dye per kgm. body weight, with specimens taken at 5 and 30 minute intervals.

Because of the findings of others (16) confirmed by the preceding paper (15) that, in acute hepatitis, a rise in gamma globulin is only demonstrable by use of the Tiselius method, comparison of the protein changes were made using both the Howe salting-out and the electrophoretic procedures. These changes in albumin and globulin by the two methods before and during malaria

are recorded in Table III and show that malarial fever induces hypoproteinemia and hypoalbuminemia. There is but slight increase in globulin by the salting-out method, whereas by electrophoresis there is considerable increase in globulin, particularly in the gamma fraction.

The electrophoretically separated albumin was studied, by the methods previously outlined (15),

TABLE II

Changes in some constituents of blood serum in a patient with Plasmodium vivax infection

After malaria (days)	Weight (lbs.)	Hours of 103° F. or over	Total hours of 103° F. or over	(Undiluted plasma) Prothrombin time (sec.)	(Diluted plasma)	Bromsulphthalein (per cent) retention 5 ^m /30 ^m	Serum Phosphatase-Alk (Bodansky units)	Serum bilirubin (mgm. per cent)	Total protein (grams per cent)	Albumin (grams per cent)	Globulin (grams per cent)	Ceph. flocculation	Cholesterol (mgm. per cent)	N.P.N. (mgm. per cent)	Uric acid (mgm. per cent)	Hemoglobin (grams)	Red blood cells (millions per c.mm.)	Total leukocytes (per c.mm.)	Erythrocyte sedimentation rate (mm. per hour)	Westergren method
0	133			21.1	42.2	95/5	3.4	0.8	7.5	5.0	2.5	0	292	37	4.6	12.5	4.5	7,000	11	
5	135	16	16						Malarial blood intravenously—Vmax											
6	135	19½	35									0	229			11.9	4.1	5,150		
7	135	21	42																	
8	132	21	49																	
10	132	21	56	27.6	64.9							2+	170	34		9.8	3.2	6,150	15	
11	134	21	61					1.8				3+	19			9.5	3.4	5,760	35	Slightly increased
12	134	5	69	17.8	45.3								127	19						
13	134	5	74	17.0	44.8								125	30	3.4					
14	134	5	69	18.9	47.1	90/25	3.2	0.6	5.2	2.9	2.3	4+	125	30		10.2	3.7	6,000	45	
15		5	74																	
16		5	81																	
17		5	81	21.4	53.1							4+	114	35		9.3	3.3	6,150	45	
18	137	4	85																	
19		4	85																	
20		3	88	18.0	45.5							4+	157	24		8.4	2.9	5,700		
21	133																			
22	129			21.6	50.4	95/35		0.4				3+	211	24		9.5	3.2	5,000	104	
23	131			22.5	60.9	95/20						4+				9.4	3.4	5,000	87	
24				22.4	49.6							3+	127			11.0	3.7	5,000		
25				22.8	43.6	90/8		0.6				1+				9.6	3.2	5,000	2	
26				19.8	42.0							0								
27												0								
28												0								
29												0								
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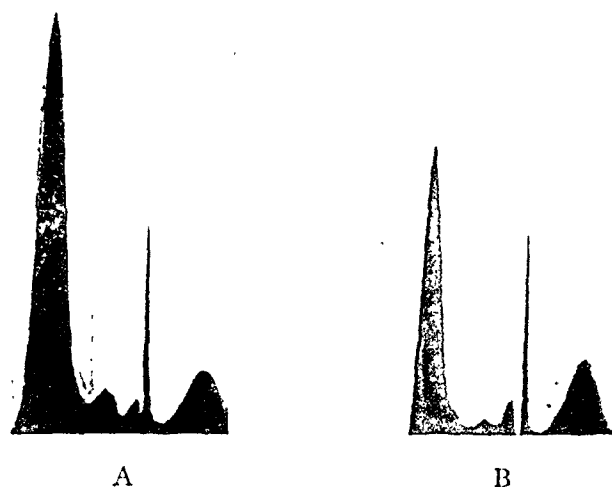


FIG. 1. ELECTROPHORESIS PATTERNS OF SERUM TAKEN FROM PATIENT No. 1

A. Before therapeutic malaria. B. During active malaria. Descending patterns. Buffer, 0.02 M sodium phosphate and 0.15 M NaCl, pH 7.4.

to determine if the changes noted in hepatitis were also present in patients with malarial fever.

Three series of 5 tubes which contained diminishing amounts of isolated gamma globulin, from 0.60 mgm. to 0.12 mgm. were prepared. In this range, the gamma globulin fraction alone gives a cephalin-cholesterol flocculation range from 4 plus to negative (17). The effect of the addition of serum albumin obtained from blood, taken both before the institution of the malarial therapy and before its termination, was determined. Striking

inhibition of flocculation occurred in all of the series of tubes containing 5 mgm. of albumin from blood taken before the institution of malarial infection. In the series of tubes, however, containing 5 mgm. of the albumin fraction from the serum of patients obtained during active malarial infection, there was but slight inhibition of flocculation in one instance (Case 1) and no inhibition in the other (Case 2) (Table IV). In the one instance tested, no differences were observed in the reaction when the gamma globulin obtained during malaria was substituted for pre-malarial gamma globulin.

DISCUSSION

The 6 patients studied were suffering from central nervous system syphilis and therefore may have had changes in their serum protein patterns some time previously. The possibility of tertiary syphilis or of anti-luetic therapy affecting the serum protein response in malaria cannot be excluded. However, all tests before the malarial induction were within normal limits, except the serological tests for syphilis.

The most consistent changes incident to the malaria shown by these studies were: (1) Decrease in serum cholesterol; (2) the development of a strongly positive cephalin-cholesterol flocculation test (1, 2); and (3) a marked hypoproteinemia, hypoalbuminemia, and an increase in globulin, especially gamma globulin. These find-

TABLE III

Electrophoretic and salt fractionation (Howe method) of blood serum before and during active Plasmodium vivax infection

Patient	Before During	Electrophoretic fractionation *							Salt fractionation—per cent			
		Total protein	Globulin					Albumin Globulin	Total protein	Albumin	Globulin	A/G
			Albumin	α	β	γ	Total					
Case I	B	7.2	5.1	0.43	0.75	1.00	2.18	2.4	7.0	5.0	2.0	2.5
	D	6.3	3.3	0.30	1.03	1.63	2.96	1.1	5.7	3.3	2.4	1.4
Case II	B	6.8	4.3	0.40	1.00	1.10	2.50	1.7	7.5	5.0	2.5	2.0
	D	5.7	2.9	0.32	1.03	1.67	3.02	1.0	5.2	2.9	2.3	1.3
Case III	B	6.8	4.5	0.47	0.83	0.87	2.17	2.0	7.1	5.1	2.0	2.5
	D	5.8	3.3	0.37	1.06	1.06	2.59	1.3	5.8	3.2	2.6	1.2

* Deduced from pattern areas assuming the same specific refractivity for all components. This introduces some error since it is known that the specific refractivity of the components vary among themselves.

TABLE IV

The effect of albumin on the cephalin-cholesterol flocculating power of gamma globulin:
a. before Plasmodium vivax infection
b. during active infection

	Gamma globulin in diminishing amounts				
	0.6 mgm.	0.48 mgm.	0.36 mgm.	0.24 mgm.	0.12 mgm.
Pre-malarial isolated gamma globulin fraction from Case I	++++	++++	+++	++	±
Pre-malarial isolated gamma globulin fraction from Case I, plus 5 mgm. pre-malarial albumin fraction from Case I	++	±	0	0	0
Pre-malarial isolated gamma globulin fraction from Case I, plus 5 mgm. post-malarial albumin fraction from Case I	+++	+++	++	±	0
Pre-malarial isolated gamma globulin fraction from Case II	++++	+++	++	±	0
Pre-malarial isolated gamma globulin fraction from Case II, plus 5 mgm. pre-malarial albumin fraction from Case II	0	0	0	0	0
Pre-malarial isolated gamma globulin fraction from Case II, plus 5 mgm. post-malarial albumin fraction from Case II	++++	+++	++	±	0

ings, if indicative of a disorder in the liver, contrast sharply with the results of other hepatic function tests such as bromsulphthalein excretion, hippuric acid synthesis, prothrombin time, and serum phosphatase values, which are often unchanged during malarial infection.

A weakly positive cephalin-cholesterol flocculation test appears early in the infection and gradually becomes strongly positive. In malaria, all three changes in the serum proteins which theoretically could produce a positive reaction (15) may account for the positive cephalin-cholesterol flocculation reaction. The marked diminution of serum albumin and the decreased inhibiting power of the electrophoretically separated albumin fraction suggest that the mechanism for synthesis of the components of the normal albumin fraction is conspicuously impaired during malarial infection. This derangement is similar to that observed in hepatitis. In the case of malaria, however, the change is more marked than other disturbances of the liver as manifested by the conventional liver function tests.

SUMMARY

1. The cephalin-cholesterol flocculation test becomes strongly positive in therapeutic malarial fever.
2. This is associated with a definite hypoproteinemia, hypoalbuminemia, and some increase in total globulin and especially in gamma globulin.
3. The serum albumin fraction during active infection shows a greatly decreased capacity to

inhibit the cephalin-cholesterol flocculating properties of gamma globulin.

4. The positive cephalin-cholesterol flocculation reaction in malaria is probably due to: (1) hypoalbuminemia, (2) a decreased capacity of the serum albumin fraction to inhibit the flocculating action of gamma globulin, and (3) an increase in the gamma globulin content of the serum in this disease.

5. These changes resemble those seen in acute hepatitis.

6. In malarial infection, the formation of serum protein is more profoundly deranged than many other functions ascribed to the liver and these changes are well demonstrated by a series of cephalin-cholesterol flocculation tests.

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COMPARATIVE STUDIES OF THE CHEMICAL CHANGES OCCURRING IN SULFONAMIDE DRUGS DURING THERAPY IN MAN

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INTRODUCTION

That sulfanilamide becomes acetylated to a considerable extent in man and in some animals has been amply demonstrated (1 to 3). This acetylation occurs on the amino group of the compound (1 to 3) to yield N⁴ acetylsulfanilamide,¹ a compound which has little if any bacteriostatic activity (2). It has since been found that, in man, all of the sulfonamide compounds now in common clinical usage become N⁴ conjugated to a greater or lesser extent (2, 5 to 11). This conjugation has been shown for most of these drugs (2, 5 to 7) to be acetylation; N⁴ conjugates with groups other than the acetyl have not been isolated.

That sulfapyridine is "detoxified" *in vivo* in the dog by chemical changes other than acetylation has recently been demonstrated (12 to 14) by the isolation of a glucuronide of a hydroxy derivative of sulfapyridine from the urine of dogs fed this drug. Also, a large increase in the urinary glucuronide excretion has been observed in man (15) and in the rat (16) following the ingestion of sulfapyridine, and some increase in the rat (16) following the ingestion of sulfathiazole.

These latter observations prompted the present study of the comparative extents to which several different sulfonamide drugs now in common clinical usage become acetylated, glucuronidated, and sulfated in man. The importance of such pharmacological studies in man resides in the fact that the therapeutic activity and toxicity of a drug depend not only upon the properties of the drug administered, but also upon the properties and the amounts of the products formed from the drug by chemical changes in the body.

¹ The nomenclature of Crossley *et al.* (4), according to which N¹ refers to substituents on the amide nitrogen of the sulfanilamide radical and N⁴ to substituents of the amino nitrogen, is used throughout this report.

MATERIAL AND METHODS OF STUDY

Material

The subjects of this investigation were 10 adult patients on the medical services of The New York Hospital, who received sulfonamide drugs in the treatment of various infections. For the purposes of study or for other reasons, as explained below in the legends to the figures, several patients received two different sulfonamide drugs at different times during the course of therapy.

In none of the subjects was there any recognized abnormal liver function. Similarly, 8 of the subjects had no recognized abnormal kidney function. Of the 2 subjects who received sulfonamide treatment for urinary tract infections, 1 (J. E. Figure 2) had only a left kidney, the right having been removed surgically for nephrolithiasis, and the other (B. K. Figure 10) had chronic pyelonephritis with pyelographic evidences of decreased excretory function in the left kidney. Neither of these 2 subjects had nitrogen retention. All of the subjects, except the 2 treated for urinary tract infections, had elevated body temperatures at the onset of sulfonamide therapy, and the therapy was continued for 2 or more days after the patient became afebrile.

Sulfonamide therapy was given orally in divided doses. No other medications which were known or suspected to affect the glucuronide or ethereal sulfate excretions were given during the course of the studies, except for the administration of acetylsalicylic acid in subject M. C. (Figure 9) on 2 days after the cessation of sulfonamide therapy. All of the patients treated with sulfadiazine, sulfamerazine, and sulfathiazole and 1 (M. C. Figure 9) treated with sulfamethazine received concomitantly 15.6 grams daily of sodium bicarbonate in divided doses as a precaution against renal complications consequent to therapy with these drugs (7).

METHODS

The urines were collected under toluol in 24-hour amounts. An aliquot was stored in the refrigerator under toluol alone, and to another aliquot similarly stored, 1 per cent of concentrated HCl was added. Free and total sulfonamide measurements were made on the unacidified specimens within a week of the urine collection. In 2 instances (J. E. Figure 2 and B. K. Figure 10), only total sulfonamide was measured because only acidified specimens stored at room temperature were available. The acidified and total specimens were used for glucuronic acid and sulfate measurements throughout.

Measurements of the concentrations of the sulfonamides in the blood and urine were made by the method of Bratton and Marshall (17). Throughout this report, the term "free" refers simply to the amount of compound, calculated on the basis of the molecular weight of the drug administered, which was diazotizable without hydrolysis, *i.e.*, had a free amino group. Similarly, the term "total" refers to the total amount of compound which was diazotizable after acid hydrolysis. That portion of the drug which became diazotizable only after hydrolysis is referred to as "N⁴ conjugated compound" (4). That "free compound" or "N⁴ conjugated compound" are not necessarily synonymous with "free drug" or "N⁴ conjugated drug" is evident from the results and discussion of the present study.

Glycuronic acid measurements were made essentially according to the method of Maughan, Evelyn, and Brown (18), which involves acid hydrolysis of the glycuronides in the urine and a color reaction between the total glycuronic acid and naphthoresorcinol. Two different samples of naphthoresorcinol purchased from Schwarz Laboratories, Inc., New York, were found readily soluble and satisfactory. It has been our practice to make up a 0.2 per cent solution of the compound in water, preserve it in a dark bottle in the refrigerator over night, and filter the next day before using. The solution so stored has kept satisfactorily for about 10 days, after which time there is considerable color development in the blank. A stock standard in water equivalent to 40 mgm. of glycuronic acid per 100 ml. was prepared from pure sodium pregnandiol glycuronide and was found to be stable in the refrigerator for at least 4 months. From this stock solution, standards equivalent to 1 mgm. and 2 mgm. of glycuronic acid per 100 ml. were made up monthly and preserved in the refrigerator. The reaction between naphthoresorcinol and glycuronic acid is slow (19). At the end of the 30-minute interval in the boiling water bath (18), only approximately 50 per cent of the complete reaction is obtained for urines or for the standards, the reaction not being complete until after approximately 3 hours in the bath. Although presumably it would be more nearly ideal to allow 3 hours of boiling, this was not found feasible due to the development of a cloudiness in the ether layer after extraction of the colored compound. Since some slight variations in the color development of the standards were found on different runs with the 30-minute boiling, we have practised including a water blank and the 1 and 2 mgm. standards with each set of unknown urines, diluted to contain from 0.5 to 1.5 mgm. of glycuronic acid per 100 ml., and have calculated the concentrations in the unknowns from the standard curve for the run, using a straight line from the origin to the point for 1 mgm. per 100 ml. It has been our practice to shake the solution for a few seconds after the addition of the alcohol, and to shake for 40 seconds with the ether to insure maximum extraction of the colored compound. The glycuronic acid values reported have been corrected for the brown contaminant in urine (18) by obtaining

colorimeter readings with both the 565 m μ and 440 m μ filter, and by applying the following equations:

$$(1) L_{T565} = L_{G565} \div L_{U565}$$

$$(2) L_{T440} = L_{G440} \div L_{U440}$$

In these equations, L_{T565} and L_{T440} are the total optical densities found with these filters, respectively, for the unknown; L_{G565} and L_{G440} are the optical densities contributed by the glycuronic acid and the urine contaminant, respectively, at 565 m μ ; and L_{U565} and L_{U440} are the optical densities contributed by these substances, respectively, at 440 m μ . Equation (2) may be converted to

$$(3) L_{T440} = G_{440:565} L_{G565} \div U_{440:565} L_{U565}$$

in which $G_{440:565}$ is the ratio of the optical densities found for pure glycuronic acid with the two filters and $U_{440:565}$ is the ratio of the optical densities found for the brown contaminant of urine with the two filters. From measurements made with standards in water, the value of $G_{440:565}$ was found to be 0.1. From spectrophotometric data (18), the value of $U_{440:565}$ was found to be 5.8. Substituting these values in equation (3), the following equation was obtained:

$$(4) L_{T440} = 0.1 L_{G565} \div 5.8 L_{U565}$$

On combining equations (1) and (4) and solving for L_{G565} , the following working equation, which gives the corrected optical density for the glycuronic acid, was obtained:

$$(5) L_{G565} = \frac{5.8 L_{T565} - L_{T440}}{5.7}$$

The corrected values were usually from 20 to 40 mgm. per day less than the values uncorrected for the 440 m μ filter reading. In almost all instances, the results reported are the average of duplicates measured on two different occasions; the results of the two analyses usually agreed well within ± 5 per cent of their average.

Measurements of urinary inorganic and ethereal sulfate were made by the benzidine titration method of Fiske (20). Approximately half of the measurements were run in duplicate; the duplicates almost always agreed within the extent of a variation of ± 1 per cent from the average calculated percentage of ethereal sulfate.

RESULTS

The dosages of the different sulfonamide drugs studied, the diseases for which they were given, and the urinary excretions of free and total sulfonamide compound, of ethereal sulfates (in percentage of total sulfate), and of glycuromides (expressed as glycuronic acid) are reported for each subject in graphic form (Figures 1 to 10 inclusive). These data, together with the average free and total blood levels of sulfonamide during ther-

TABLE I
Summary of the data of this study

Patient and figure number	Sex	Drug administered	Daily dosage of drug during study	Number of days studied during therapy	Average blood level of sulfonamide compound			Average urinary excretion of sulfonamide compound			Average daily total sulfate excretion		Average etheral sulfate excretion		Average daily glycuronate excretion	
					Free	Total	N ⁴ conjugated	Free	Total	N ⁴ conjugated	During control	During therapy	During control	During therapy	During control	During therapy
			grams		mgm. per 100 ml.		per cent	grams		per cent	grams sulfur		per cent of total sulfate		mgm. glycuronic acid	
H. W. Fig. 1	♀	Sulfanilamide	7 to 3 (av. 5.5)	4	12.3	15.2	19	2.49	3.94	38	0.67	0.53	4	9	395	340
J. E. Fig. 2	♂	Sulfadiazine	4	28	7.2				2.85						505	535
L. L. Fig. 3	♂	Sulfadiazine	6	6	6.5			3.63	4.98	27					525*	480
A. H. Fig. 4	♀	Sulfapyrazine	12	6	3.8	4.3	12	2.96	3.91	24	0.77	0.60	7	9	625	625
O. P. Fig. 5	♂	Sulfathiazole	6	3	6.0	6.5	8	4.47	5.28	15	0.69	0.57	4	7	470	630
B. D. Fig. 6	♀	Sulfadiazine	6	2	8.5			4.20	5.02	16	0.43	0.30	5	7	445	465
		Sulfamerazine	6	2	16.7	17.3	3	3.81	5.52	31	0.43	0.34	5	12	445	895
M. R. Fig. 7	♀	Sulfamerazine	6	2	16.5	17.6	6	3.20	4.88	34	0.60	0.64	6	6	480	1290
			3	7	10.2	11.1	8	1.66	2.67	37	0.60	0.59	6	5*	480	925
R. N. Fig. 8	♂	Sulfapyridine	4 to 6 (av. 5.3)	4	13.0	14.9	13	2.30	3.79	39	0.54	0.95	9	9	535	1670
		Sulfadiazine	8 to 6 (av. 6.2)	8	8.2			3.82	4.45	14					535	510
M. C. Fig. 9	♀	Sulfamethazine	6	34	5.1	9.4	46	1.34	6.00	78	0.87	0.38	5	6	605	1210
		Sulfadiazine	6	4	8.1	8.9	9	4.02	5.12	21	0.87	0.74	5	8	605	760
B. K. Fig. 10	♂	Sulfamethazine	4	6	6.9	8.6	20		2.93						500	1800

* Only 1 day of study.

apy and in the total sulfate excretion, are summarized in Table I.

Blood and urinary free and total sulfonamides. The daily amount of total sulfonamide excreted in the urine by these patients ranged between 71 and 100 per cent of the daily dosage for all of the drugs studied except sulfapyrazine, where the urinary output of total compound was only 33 per cent of the intake (Figure 10).

The percentage of N⁴ conjugated compound excreted was less than 30 per cent for sulfadiazine, sulfathiazole, and sulfapyrazine, from 30 to 40 per cent for sulfanilamide and sulfamerazine, and 78 per cent in a patient receiving sulfamethazine (Figure 9). The difference in the tendency of a given drug to become N⁴ conjugated is shown particularly well in the studies where two drugs were administered at different times to the same individual. Thus in R. N. (Figure 8), 39 per cent of the excreted compound was N⁴ conjugated during therapy with sulfapyridine compared with only 14 per cent during sulfadiazine therapy. Similarly in B. D. (Figure 6), 31 per cent of the excreted compound was N⁴

conjugated during therapy with sulfamerazine and only 16 per cent with sulfadiazine. Most striking was the finding in M. C. (Figure 9) that 78 per cent of the excreted compound was N⁴ conjugated during sulfamethazine therapy, as against only 21 per cent during subsequent sulfadiazine therapy. This tendency for sulfamethazine to become N⁴ conjugated to a great extent is also obvious in the findings that, in the two patients treated with this drug, averages of 20 and 46 per cent of the blood sulfonamide compound were not diazotizable until after acid hydrolysis (Table I).

Urinary etheral sulfate excretion. Both during sulfonamide therapy and control periods, the daily amount of total sulfate excreted in the urine was variable in different individuals, and in the same individual on different days, as was to be expected since the dietary intakes were not controlled. The differences in the average daily urinary excretions of total sulfate in a given individual were not so great between periods of therapy and control (Table I) as to affect appreciably the percentage of sulfate to be expected in the form of etheral sulfate (21). Neither the

amount of ethereal sulfate nor the percentage of total sulfate excreted as ethereal sulfate in the urine was consistently increased above normal during therapy with any of the drugs of this study. In two instances, H. W. (Figure 1) and B. D. (Figure 6), the values for the percentages of total sulfate excreted as ethereal sulfate during periods of therapy with sulfanilamide and sulfamerazine, respectively, were approximately twice the values obtained during the respective control periods. The authors do not believe that these differences necessarily indicate that a portion of these sulfonamides became sulfated. It is to be

noted that in another subject, M. R. (Figure 7), who received sulfamerazine, no increased ethereal sulfate excretion was observed during this therapy (Table I). If the increase in ethereal sulfate were interpreted as indicating that a sulfated sulfonamide compound had been formed, calculations from the data for the increased amount of ethereal sulfate excreted (Table I) and from the molecular weights of the drugs administered and the atomic weight of sulfur show that only 0.11 gram of the daily excretion of 3.94 grams of total sulfanilamide in H. W. (Figure 1), and only 0.16 gram of the daily excretion of 5.52 grams

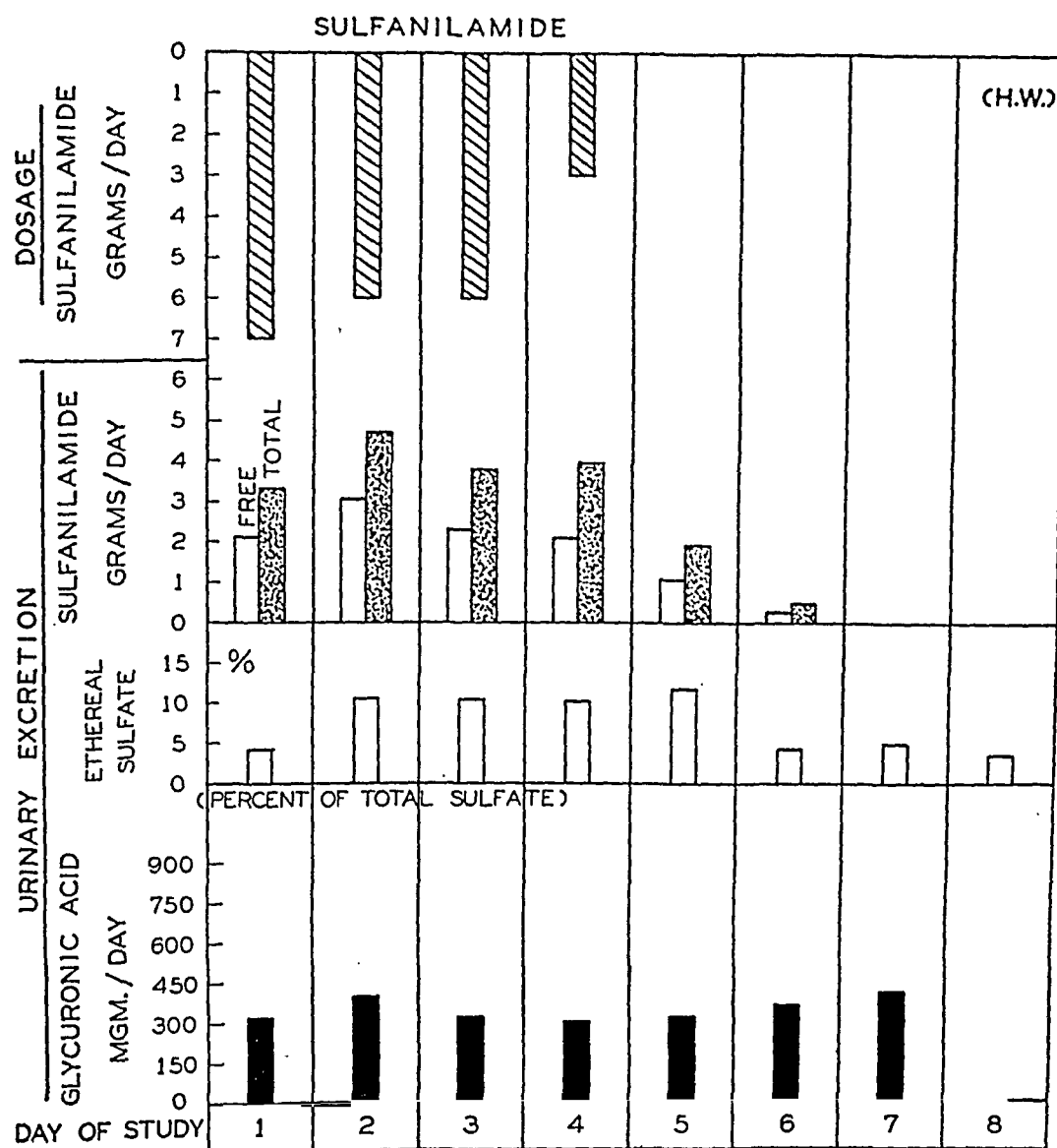


FIG. 1. SULFANILAMIDE

Patient H. W., a girl aged 13, received 7 to 3 grams (av. 5.5 grams) of sulfanilamide daily for 4 days in the treatment of a beta hemolytic streptococcus infection of the throat. The excretion studies started with the initiation of sulfanilamide therapy.

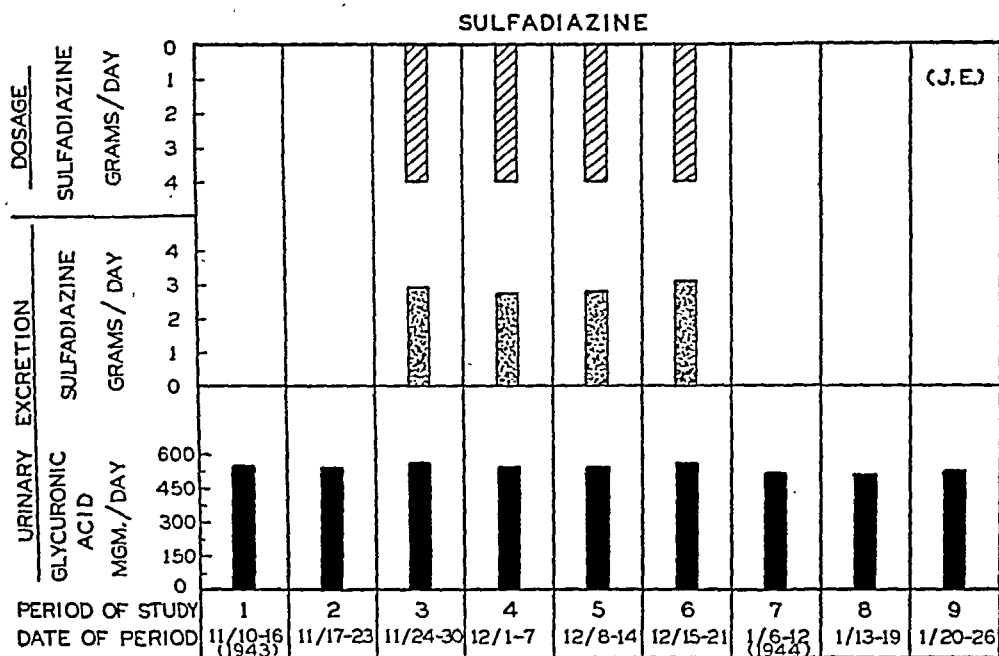


FIG. 2. SULFADIAZINE

Patient J. E., an adult male, received 4 grams of sulfadiazine daily for 28 days in the treatment of a urinary tract infection with non-hemolytic streptococcus, gamma, and *B. lactis acrogens*. The urines from this patient were combined for study* in 7-day periods. Studies were made on 2 control periods just prior to sulfadiazine therapy, on 4 periods of sulfadiazine therapy, and on 3 control periods which began approximately 2 weeks after the cessation of therapy.

* We are indebted to Dr. Ephraim Shorr for affording us the opportunity to study these specimens collected by him on the Research Metabolism ward of The New York Hospital, in affiliation with the Russell Sage Institute of Pathology, for the purpose of other studies. The urines in this instance had been preserved for several months with sulfuric acid so that the percentage of N⁶ conjugated sulfonamide was not measurable because of interim hydrolysis. Sulfate excretion studies also were not possible because of the addition of the sulfuric acid.

of total sulfamerazine in B. D. (Figure 6) were excreted in this form.

Urinary glycuronide excretion. The urinary glycuronide excretion (measured as glycuronic acid) was normal during sulfanilamide therapy in the 1 patient studied (Figure 1), during sulfadiazine therapy in 4 patients (Figures 2, 3, 6, 8), and during sulfapyrazine therapy in 1 patient (Figure 4) (Table I). In the 1 study with sulfathiazole, the glycuronide excretion, although not above the upper limit of normal excretion which may be encountered, was definitely and consistently higher during therapy than after its cessation (Figure 5, and Table I). The glycuronide excretions were strikingly increased above normal in 1 patient during therapy with sulfapyridine (Figure 8), in 2 patients while receiving sulfa-

merazine (Figures 6, 7), and in 2 patients while receiving sulfamethazine (Figures 9, 10); in each of these patients the glucuronide excretion returned to normal values on cessation of sulfonamide therapy. Again, the differences in glucuronide excretion encountered with different sulfonamide drugs are particularly well demonstrated in instances where two drugs were given at different times during therapy in the same patient. Thus, in R. N. (Figure 8), the glucuronide excretion fell from the very high levels obtained during sulfapyridine therapy to normal during subsequent sulfadiazine therapy. In B. D. (Figure 6), the glucuronide excretion was normal during sulfadiazine therapy but increased strikingly when therapy with this drug was replaced by sulfamerazine. In M. C. (Figure 9), the glucuronide

excretion was strikingly increased during sulfamethazine therapy but decreased toward normal when the therapy was changed to sulfadiazine.

It is of note that the percentage of sulfonamide compound excreted as a glucuronide increased gradually during the first 3 days of sulfamethazine therapy in M. C. (Figure 9) and of sulfapyridine therapy in R. N. (Figure 8). Similarly, the percentage of compound excreted in the N^4 conjugated form gradually increased in these subjects.

From calculations based on the assumption that the glucuronides excreted during therapy with sulfathiazole, sulfapyridine, sulfamerazine, and sulfamethazine were monoglucuronides, it appears that the percentage of the total sulfonamide excreted as a glucuronidated compound varied from 4 per cent in a patient receiving sulfathiazole to 68 per cent in a patient receiving sulfamethazine (Table II).

DISCUSSION

The studies of the present investigation were made directly in man since it is well known that the processes of "detoxication" of foreign substances may vary greatly in different species (22). That this is true in respect to the sulfonamides is demonstrated by the fact that certain of these compounds which become N^4 acetylated in man (2, 5, 6) also become N^4 acetylated in the rabbit and mouse (2) but do not undergo this chemical change in the dog (1, 14). On the other hand, in the dog (12 to 14), and in the rat (16), as well as in man (15) (Figure 8), the urinary excretion of glucuronide is greatly increased by sulfapyridine administration. In the rat (16) as well as in man (Figure 5), there is some increased glucuronide excretion with sulfathiazole, but no increase in either the rat (16) or man

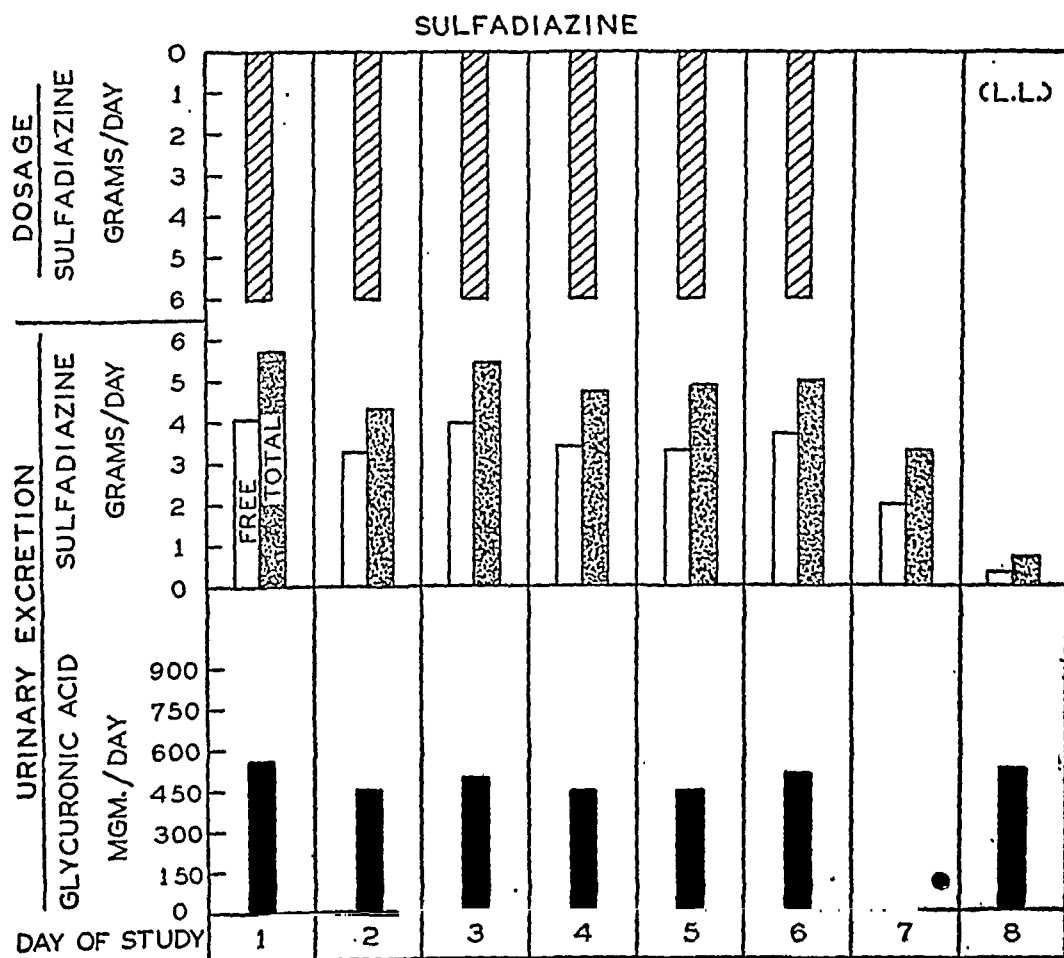


FIG. 3. SULFADIAZINE

Patient L. L., an adult male, received 6 grams of sulfadiazine daily for 6 days in the treatment of pneumococcal pneumonia. The studies were begun during the 1st day of therapy.

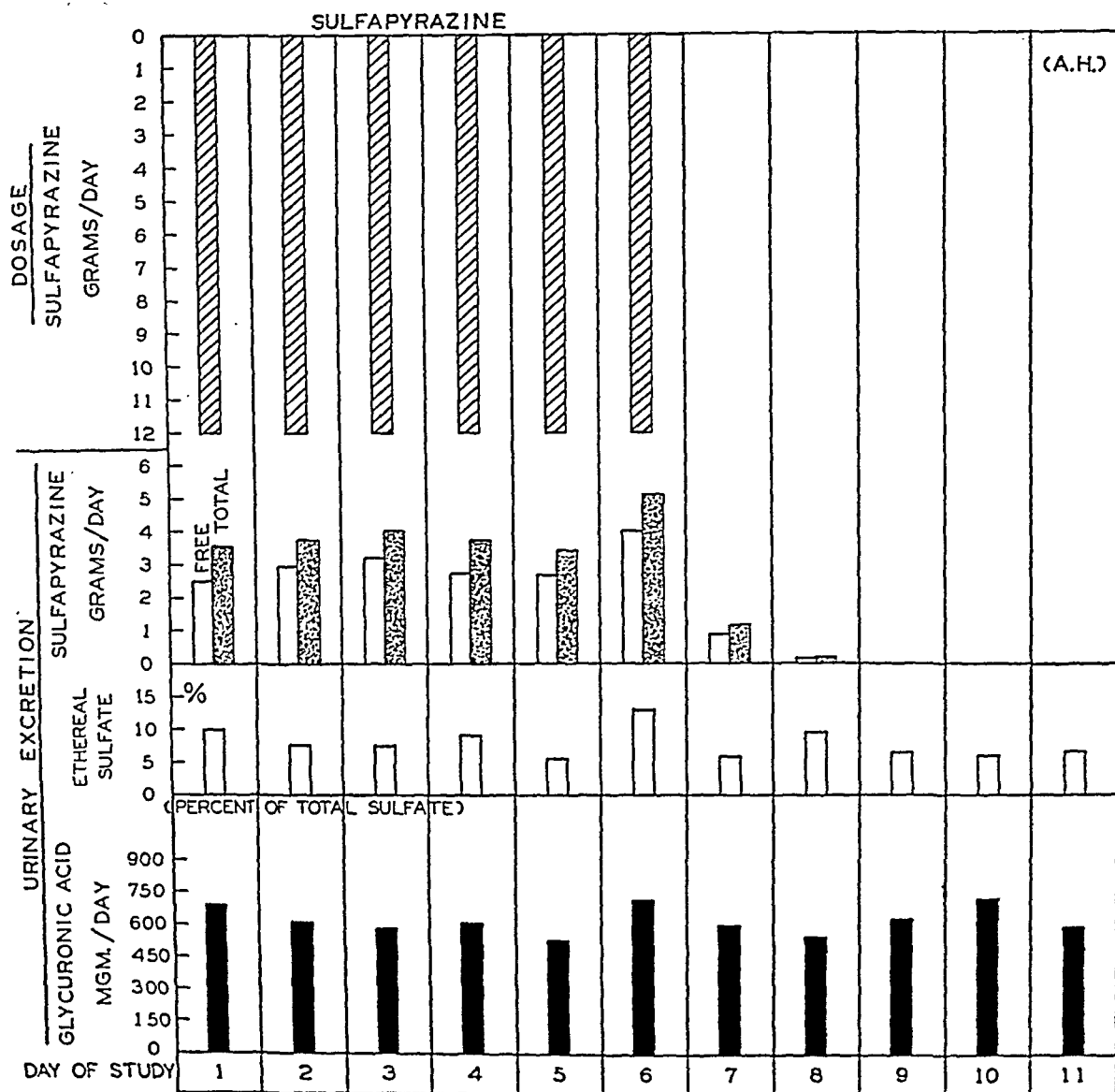


FIG. 4. SULFAPYRAZINE

Patient A. H., an adult female, received 6 grams of sulfapyrazine for 1 day, followed by 12 grams of this drug daily for 6 days in the treatment of pneumococcal pneumonia. The dosage of sulfapyrazine was increased to 12 grams on the 2d day of therapy because the blood level of "free" sulfapyrazine was only 28 mm. per 100 ml. after 1 day of treatment with 6 grams of the drug. This study was begun on the 2d day of treatment.

(Figure 1) with sulfanilamide. Again, although no increase above normal in the etheral sulfate excretion was found in our patient treated with sulfanilamide (Figure 1), a considerable increase in the etheral sulfate excretion in rabbits after the ingestion of this drug has been reported (23).

Although it has been previously demonstrated that all of the sulfonamides studied in this inves-

tigation become N^4 conjugated to varying extent in man, and that this N^4 conjugation is probably in nature always acetylation (1, 3, 5 to 7), it has not been shown previously that any of these compounds except sulfapyridine (15) undergo chemical changes in man which lead to conjugation other than this N^4 acetylation.

The findings of the present study confirm as

TABLE II

Calculated percentages of urinary sulfonamides excreted as glycuronides (mono) as compared with percentages excreted as N⁴ acetyl derivatives in the patients treated with sulfathiazole, sulfapyridine, sulfamerazine, and sulfamethazine

Patient and figure number	Drug administered	Average dosage of sulfonamide	Sulfonamide compound excreted		Increase in urinary glycuronic acid over control	Total sulfonamide excreted as N ⁴ acetyl derivative	Total sulfonamide excreted as monoglycuronide
			Free	Total			
		<i>grams per day</i>	<i>grams per day</i>		<i>mgm. per day</i>	<i>per cent</i>	
O. P. Fig. 5	Sulfathiazole	6	4.47	5.28	160	15	4
B. D. Fig. 6	Sulfamerazine	6	3.81	5.52	450	31	12
M. R. Fig. 7	Sulfamerazine	6	3.20	4.88	810	34	24
M. R. Fig. 7	Sulfamerazine	3	1.68	2.67	445	37	24
R. N. Fig. 8	Sulfapyridine	5.3	2.30	3.79	1115	39	41
M. C. Fig. 9	Sulfamethazine	6	1.34	6.00	605	78	16
B.K. Fig. 10	Sulfamethazine	4		2.93	1300		68

quantitate the previous observation (15) that in man, the urinary glycuronide excretion is increased during the excretion of sulfapyridine, and further demonstrate that in man the urinary glycuronide excretion is also greatly increased during therapy with sulfamerazine and sulfamethazine, significantly increased with sulfathiazole, and not observably increased with sulfanilamide, sulfadiazine, and sulfapyrazine.

The products of the conjugation of these sulfonamides with glycuronic acid have not as yet been isolated from the urine of man and identified. The *in vivo* glycuronic acid derivative of sulfapyridine, isolated from the urine of dogs after sulfapyridine administration, has been demonstrated to be a glycuronic acid conjugate of an hydroxy derivative of the pyridine component of this drug (13, 14). It is of interest that although sulfadiazine itself does not lead to increased glycuronic acid excretion in man (Table I), when one methyl group is substituted in the 4-position of the pyrimidine radical of sulfadiazine or two methyl groups are substituted in the 4-6 positions, the resulting drugs, namely sulfamerazine and sulfamethazine, respectively, do lead to greatly increased glycuronic acid excretion. There is evidence (22) that the products of the methylation of certain heterocyclic hydrocarbons other than pyrimidines undergo much greater or quite different chemical changes *in vivo* than do the parent compounds.

In vivo studies (24) indicate strongly that N⁴

acetylation of sulfanilamide occurs in the liver and not in any other tissue. Much evidence has been presented that with certain compounds other than sulfonamides, glycuronidation similarly takes place in the liver, and probably only in the liver (22). Further strong indication that glycuronidation of sulfonamide compounds occurs in the liver is the observation (16) that in rats with liver damage by phosphorous poisoning, the glycuronic acid excretion fails to increase after the ingestion of sulfapyridine.

With none of the drugs studied in this investigation was there any evidence of the formation *in vivo* in man of sulfated sulfonamide compounds. That glycuronidation takes precedence in man over sulfate formation with certain hydroxy compounds not of the sulfonamide group has already been demonstrated (25).

It is of interest that sulfapyridine, sulfamerazine, and sulfamethazine which become acetylated in man to an irregular and relatively large extent also become glycuronidated to a considerable extent (Table II), whereas sulfanilamide, sulfadiazine, and sulfathiazole which acetylate to a lesser extent show no or a very slight (sulfathiazole) tendency to glycuronidate. No general correlation between acetylation and glycuronidation is warranted since sulfapyrazine which acetylates irregularly in different patients and sometimes to a large extent (9) showed no tendency to glycuronidation in the patient of our study (A. H. Figure 4). It is of further interest that,

whereas sulfadiazine becomes acetylated to a relatively small extent and does not become glucuronidated (Table I), its mono and di-methyl derivatives, sulfamerazine and sulfamethazine, respectively, become acetylated to a much larger and more variable extent and also become glucuronidated, the degree of these chemical changes appearing to be greater with the dimethyl derivative (Tables I and II).

Since a very large portion of the therapeutic dose of a sulfonamide drug may become chemically changed in the human body, and since the

processes of "detoxication" vary greatly in different species, it is evident that studies of the *in vitro* bacteriostatic activity of any such drug and of its therapeutic and toxic properties in animals serve only to indicate the advisability of clinical trial. Thus, although sulfamethazine has approximately the same bacteriostatic activity *in vitro* (26) as do sulfadiazine and sulfathiazole and has been judged highly active against organisms *in vivo* in mice (10), it has not proved to be as reliable a therapeutic agent in man as are sulfadiazine and sulfathiazole (11, 27) in the same

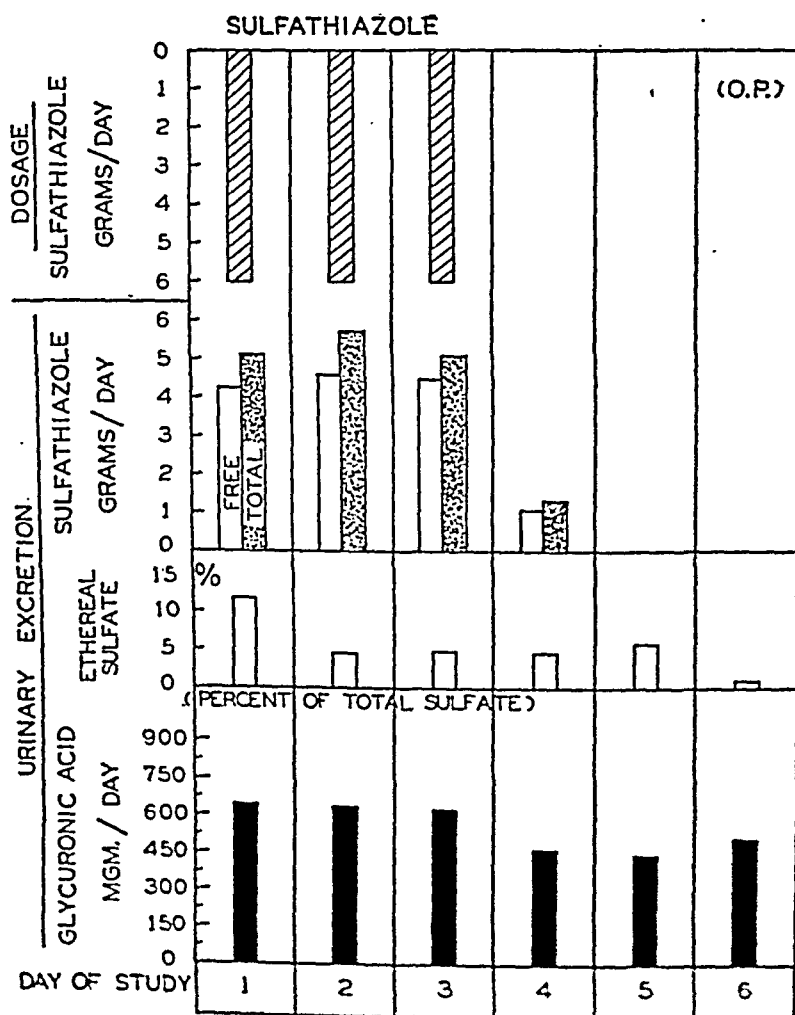


FIG. 5. SULFATHIAZOLE

Patient O. P., an adult male, received 6 grams of sulfathiazole daily for 4 days in the treatment of a beta hemolytic streptococcus infection of the throat. The excretion studies were begun on the 2d day of treatment.

dosage. On the basis of earlier studies (11), it was suggested that this lesser therapeutic value of sulfamethiazine was attributable to the fact that this drug became N^4 acetylated to a large extent in a considerable number of patients, thereby losing a large part of its bacteriostatic activity. It may also be that of the portion of free compound remaining in the body a considerable amount may be glucuronidated. Although the data of this study do not afford direct infor-

mation as to the exact nature of the glucuronidated compound, it is of interest that in M. C. (Table I), in whom sulfamethiazine was very highly N^4 acetylated, the calculated amount excreted as a glucuronide (Table II) was much less than in B. K. (Table I) who acetylated the drug to a much lesser degree. These findings possibly indicate that glucuronidation and N^4 acetylation do not occur in the same molecule.

It has been previously demonstrated (22) that

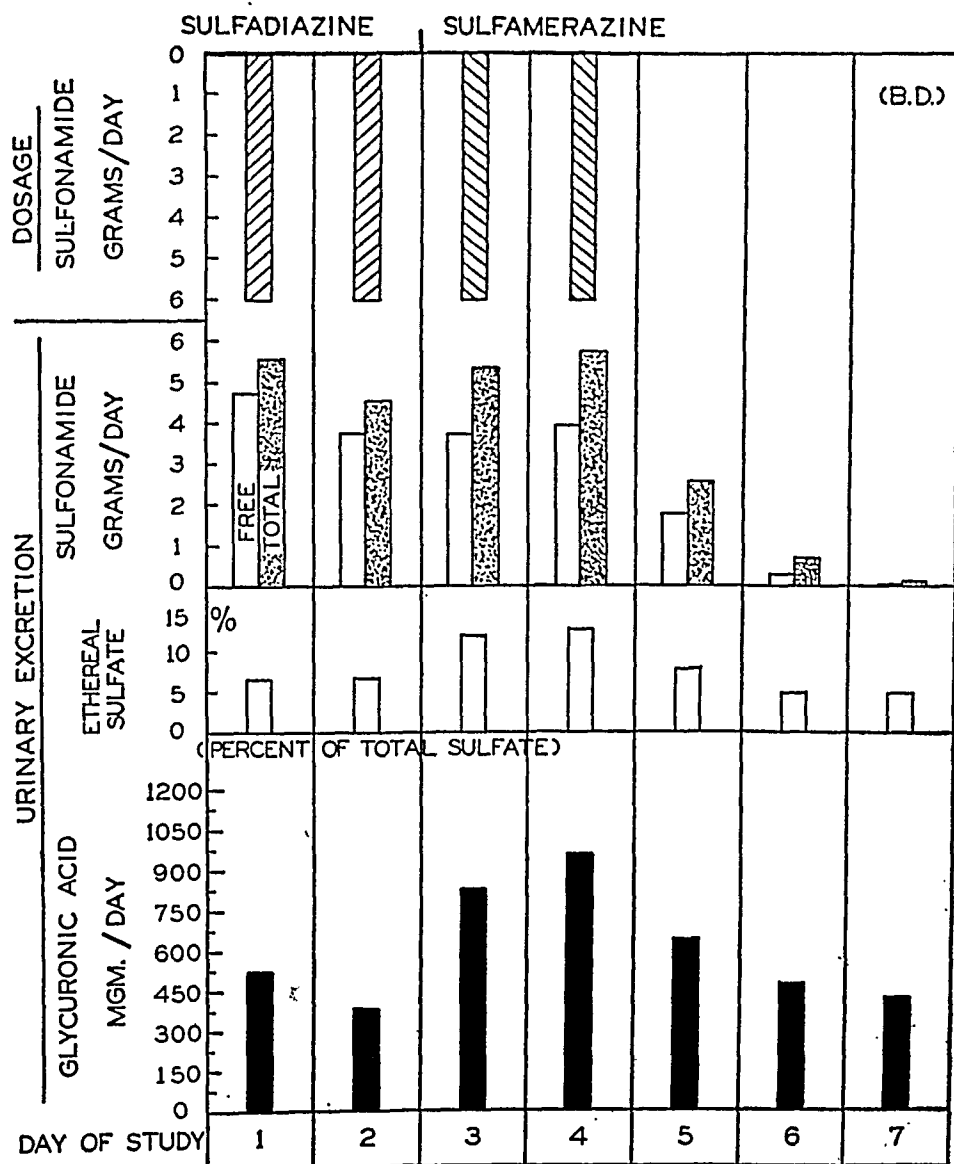


FIG. 6. SULFADIAZINE, SULFAMERAZINE

Patient B. D., an adult female, received 6 grams of sulfadiazine daily for 3 days and 6 grams of sulfamerazine daily for the following 2 days in the treatment of pneumococcal pneumonia. Sulfadiazine was replaced by sulfamerazine on the 4th day of treatment in order to compare the findings of this study in the same subject with the two different drugs. The study was begun on the 2d day of sulfadiazine therapy.

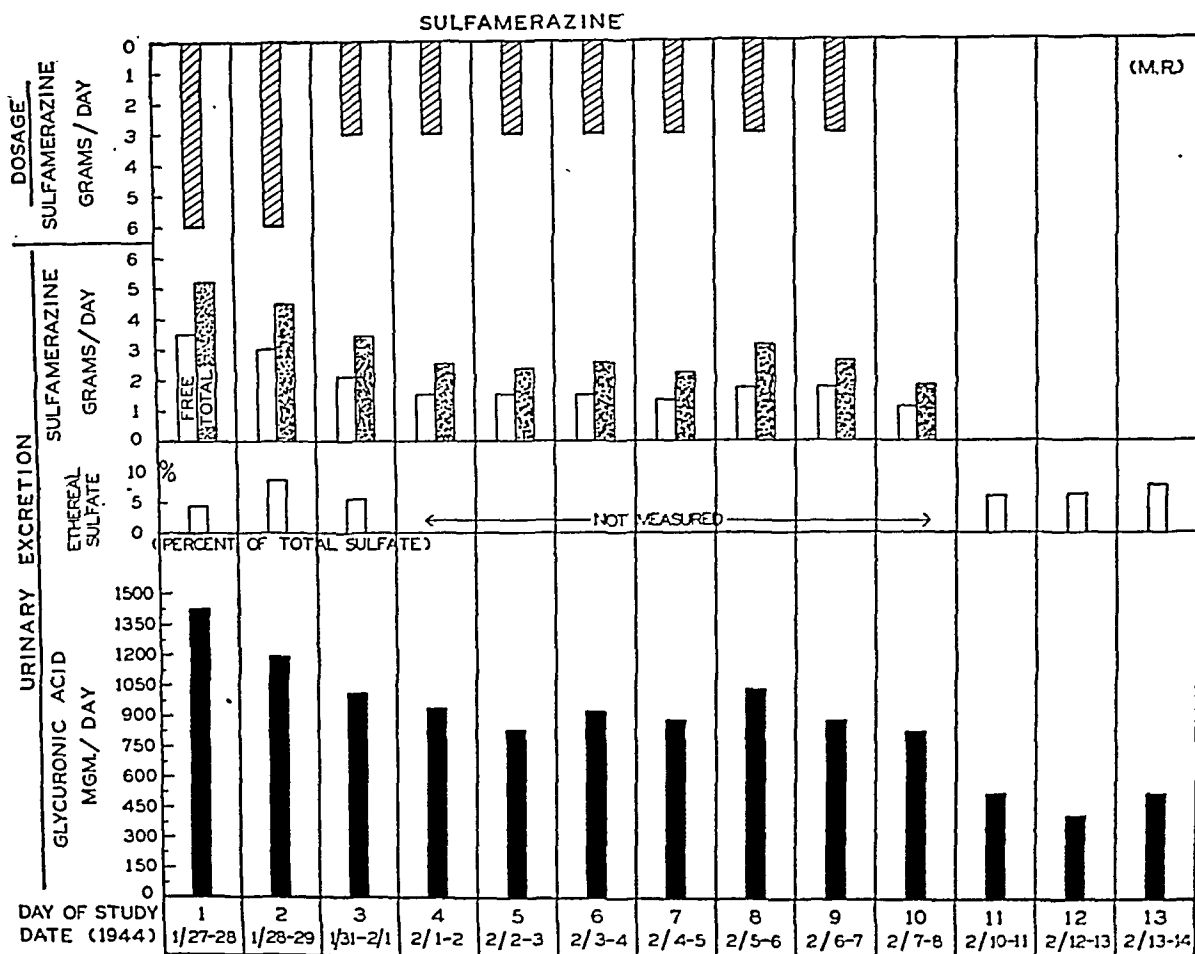


FIG. 7. SULFAMERAZINE

Patient M. R., an adult female, received 6 grams of sulfamerazine daily for 3 days and 3 grams of this drug daily for the following 9 days in the treatment of pneumococcal pneumonia and otitis media. The study was begun on the 2d day of therapy.*

* Urine collections from this patient were known to be incomplete on the 1st and 2d days of therapy with 3 grams of sulfamerazine, and on certain days during the control period. These days are indicated on the figure by the lack of complete continuity of the dates of study.

some foreign compounds, other than sulfonamides, which are not necessarily intrinsically toxic, owe their toxicity in part or in whole to those very *in vitro* reactions which lead to the formation of so-called "detoxication" products. That the *in vitro* N⁴ acetyl derivatives of several of the sulfonamide drugs in common usage may give rise to renal complications because they precipitate in the kidneys and urinary tract is well appreciated (6, 7). Whether the sulfonamide drugs administered or one of their *in vitro* derivatives

gives rise to the febrile and skin reactions and the hematopoietic disturbances not infrequently observed is not known. That minute amounts of foreign compounds are sufficient to cause toxic reactions in man is well established. It has been suggested (28) that the cyanosis occurring during sulfanilamide therapy is due to the *in vitro* formation of an oxidizing agent by sulfanilamide.

It is to be pointed out that any renal clearance studies of sulfonamide compounds which are to have exact significance must be based on separate

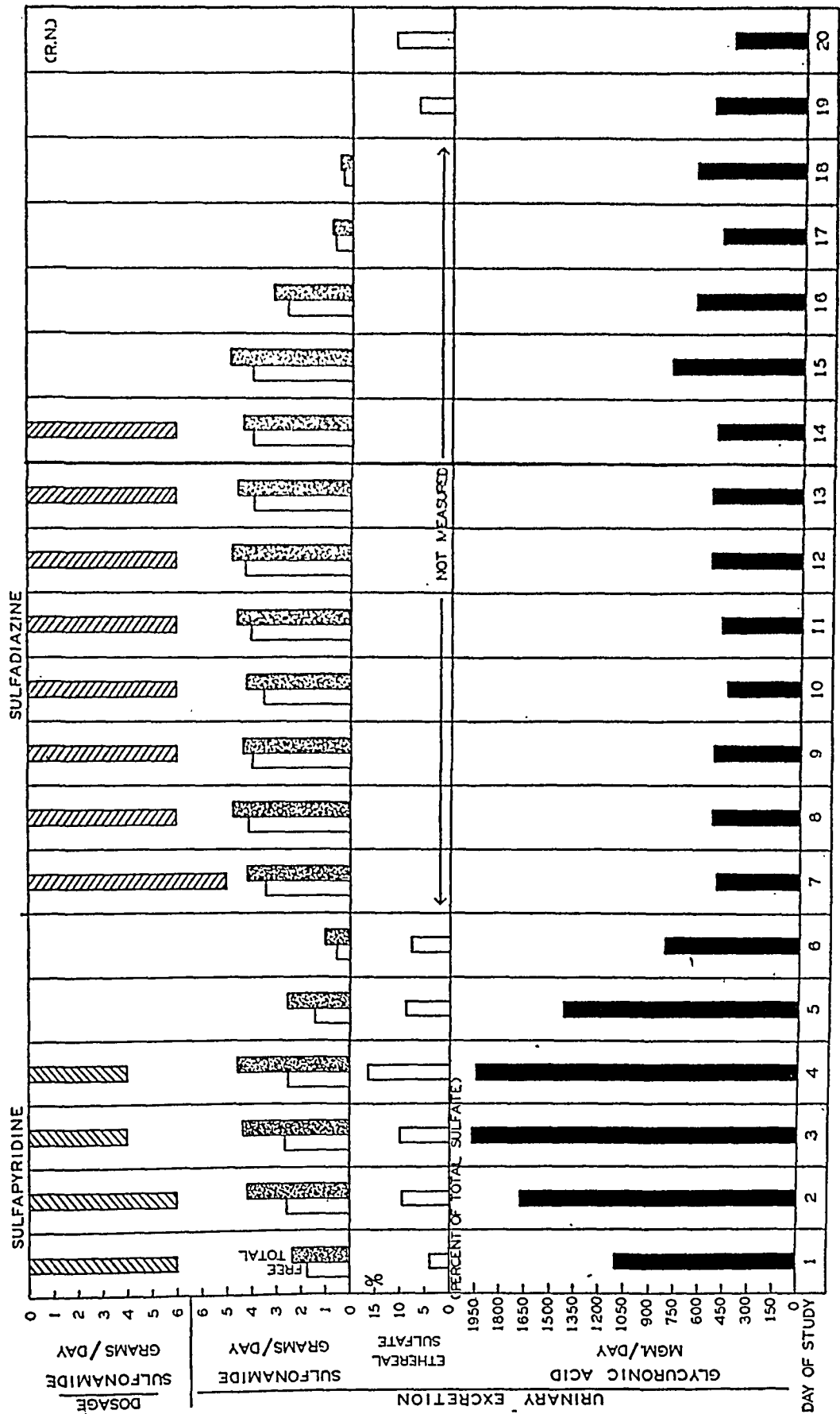


FIG. 8. SULFAPYRIDINE, SULFADIAZINE

Patient, R. N., an adult male, received 6 to 4 grams of sulfapyridine daily for 4 days in the treatment of pneumococcal pneumonia. Because of recurrence of signs and symptoms of pneumonia on the 3d day following cessation of sulfapyridine therapy, a course of therapy with 6 grams of sulfadiazine was given daily for 8 days. The study was begun during the 1st day of treatment with sulfapyridine.

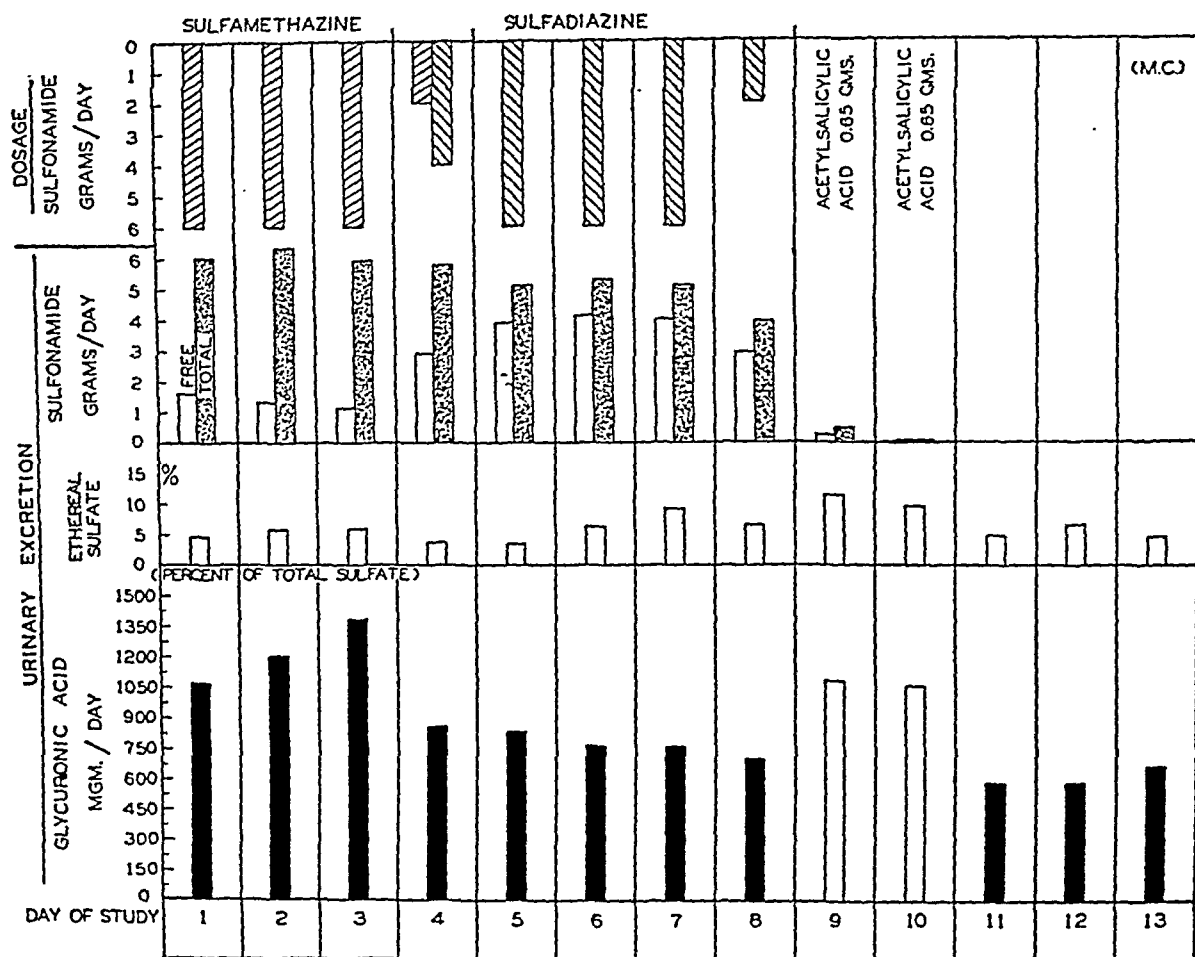


FIG. 9. SULFAMETHAZINE, SULFADIAZINE

Patient M. C., an adult female, received 6 grams of sulfamethazine daily for 4 days in the treatment of pneumococcal pneumonia. The study was begun during the 1st day of treatment. Sulfadiazine was given in place of sulfamethazine on the 5th day of treatment because the clinical response with the latter drug had not been satisfactory. The change in therapy afforded an opportunity for this comparative study of the fate of the two drugs. On the 9th and 10th days of study, 0.65 gram of acetylsalicylic acid was administered to the patient for the relief of pain from myositis. (N.B.—The glycuronide output was increased considerably during the acetylsalicylic acid medication.) This study was begun during the 1st day of sulfamethazine therapy.

studies of the several different compounds which may be present in the blood and urine of the particular species studied.

SUMMARY AND CONCLUSIONS

1. To gain information concerning the chemical changes which sulfonamide drugs undergo in man, studies of the urinary excretion of free and total sulfonamide compounds, of glycuronide, and of etheral sulfates have been made in patients during and following therapy with sulfanilamide,

sulfapyridine, sulfathiazole, sulfadiazine, sulfapyrazine, sulfamerazine, and sulfamethazine.

2. The results confirm previous reports showing the variability in the extent to which these drugs become N^4 acetylated in man, sulfadiazine and sulfathiazole being the least acetylated and sulfamethazine the most in the subjects studied.

3. The etheral sulfate excretion was not consistently increased above normal during therapy with any of these drugs. The data are interpreted to show that no appreciable amount of any

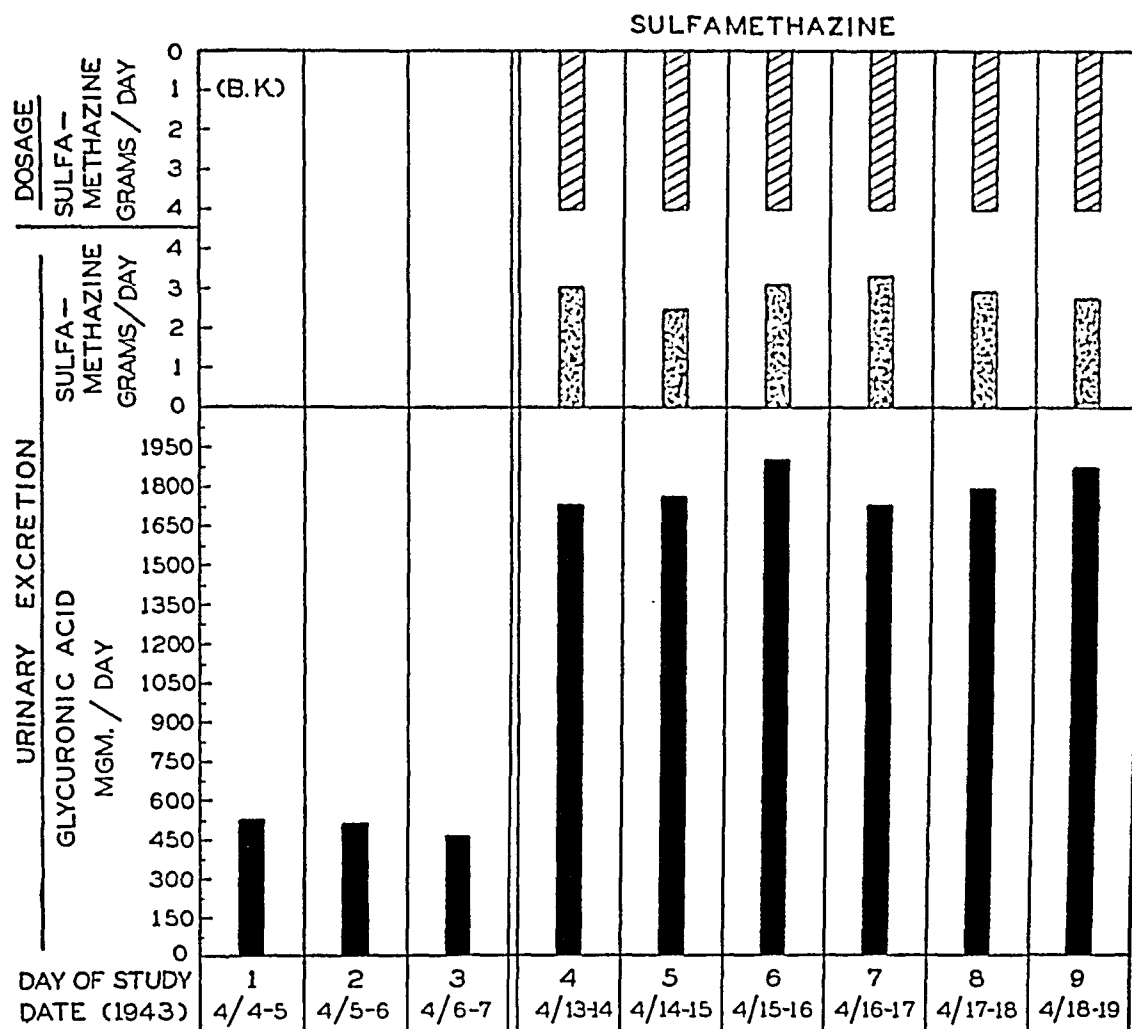


FIG. 10. SULFAMETHAZINE

Patient B. K., an adult male, received 4 grams of sulfamethazine daily for 12 days in the treatment of a urinary-tract infection with *Staphylococcus aureus*. Studies were made of the glycuronic acid excretion during 3 control days before sulfamethazine therapy was instituted and during the last 6 days of therapy when the patient was receiving 4 grams of the drug daily.*

* See footnote in legend of Fig. 2.

of these drugs or of any of their *in vivo* derivatives in man becomes sulfated.

4. The glycuronide excretion was not increased during therapy with sulfanilamide, sulfadiazine, or sulfapyrazine, was slightly increased with sulfathiazole, and was greatly increased during therapy with sulfapyridine, sulfamerazine, and sulfamethazine. These findings are interpreted as due to the formation of glycuronidated sulfonamide compounds *in vivo*.

5. Calculations based on the assumption that a monoglycuronide is formed *in vivo* show that from 4 per cent (with sulfathiazole) to 68 per

cent (with sulfamethazine) of the total sulfonamide compounds excreted in the urine during therapy with sulfathiazole, sulfapyridine, sulfamerazine, and sulfamethazine were glycuronidated.

6. The findings have been discussed in relation to their bearing on the interpretation of *in vitro* studies of sulfonamide drugs, of *in vivo* studies in animals, and of existing data on the renal clearance of these drugs.

7. It has been pointed out that the toxicity and therapeutic value of a sulfonamide drug in man depend not only on these properties of the drug administered but on the properties of any of the

several compounds which may be formed from the drug *in vivo*.

We wish to express our appreciation to Drs. Ephraim Shorr and Robert Furchgott for their many helpful suggestions during the progress of this work.

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FACTORS INFLUENCING ASCITES IN PATIENTS WITH CIRRHOSIS OF THE LIVER¹

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The level of serum albumin has been said to be the controlling factor in the accumulation of ascitic fluid in patients with cirrhosis of the liver (1 to 4). Several investigators have reported an increased portal pressure in patients with the disease (5 to 7) and this has been conceded to contribute to the development of ascites. During the past 3 years, we have observed patients with cirrhosis of the liver who have accumulated ascitic fluid in such quantities that repeated paracenteses were required. The plasma levels of albumin and globulin were determined at regular intervals. The patients received, in addition to an adequate diet, an aqueous extract of liver prepared at the Rockefeller Institute for Medical Research. The liver extract, diluted with saline, was administered intravenously. As a result of the combined therapy, the reaccumulation of ascitic fluid was arrested after varying periods of time in the different patients and further paracenteses were unnecessary. We were thus able to compare the plasma levels of albumin and globulin during and after the period of fluid retention. In addition, determinations of plasma proteins were made on a group of patients with severe cirrhosis of the liver in whom ascites had never been present.

This report is concerned with these observations. As the results did not indicate that the level of albumin in the plasma was the determining factor in the fluid retention, data are presented to support another theory in explanation of this disturbance.

STUDIES ON PATIENTS

The patients selected for this study were those in whom severe liver damage was unquestionably present

¹ This research was aided by grants from the Milbank Memorial Foundation and from the Nutrition Foundation.

as evidenced by the physical findings and laboratory tests. A history of chronic alcoholism extending over a period of years was given by each patient. During the period of hospitalization, the patients were on the wards of the 3rd (New York University) Medical Division of Bellevue Hospital. When discharged, they were seen regularly in the Metabolism Clinic of New York University College of Medicine. The diet consisted of 450 grams of carbohydrate, 100 to 120 grams of protein, and 85 grams of fat. Meat was given only once a day and the rest of the protein was derived from milk, eggs, cheese, and gelatine.² The liver extract³ was given intravenously in doses of 5 to 10 ml. diluted with 40 ml. of N/saline. Two to 3 injections were given weekly. The plasma levels of albumin and globulin were determined by the method of Howe (8). The total proteins were done by the micro Kjeldahl method. Plasma cholesterol was determined by a modification of the Schoenheimer and Sperry method (9), and were read in the photoelectric colorimeter (10). Five mgm. of bromsulphalein per kgm. of body weight were injected intravenously for this test and the retention was determined on a sample of plasma withdrawn after one-half hour. The readings were made in a photoelectric colorimeter. The colloid osmotic pressure was calculated by the formula of Wies and Peters (11) with corrections made for the water content of the plasma using Eisenman's formula (12).

Six patients in whom severe ascites was arrested have been observed for periods of 3 months to 2½ years. Two patients with severe ascites died during the periods of study. One patient observed for 7 months is still accumulating ascitic fluid. Four patients had minimal or no ascites but had enlarged livers and evidences of

² Knox Gelatine, Charles B. Knox Gelatine Co., Johnstown, N. Y.

³ The liver extract used in this study was made from Lilly's 343, dried, powdered extract, by hot aqueous extraction. The extract was filtered through a Permutit tower, and the filtrate autoclaved at pH 8.3 for 20 minutes at 116° C. The pH was then adjusted to 7.2 and the material again filtered. The filtered extract was tested for sterility and toxicity, bottled in ampoules, and stored in a cool place until used. Full details concerning the preparation of this extract will appear in another communication.

severe hepatic damage as shown by liver function tests and the albumin levels in the plasma.

RESULTS ON PATIENTS

In Table I are recorded the clinical data, the results of the bromsulphalein tests, and the cholesterol determinations. The levels of albumin and globulin in the plasma during the period of ascites and at varying intervals after the cessation of ascites are given in Table II, along with the calculated colloid osmotic pressure. Table III gives the albumin and globulin levels at different intervals in the patients in whom ascites was not present clinically.

In all the patients, the bromsulphalein retention was increased, and the ratio of free/total cholesterol was significantly greater than in the normal. In both groups of patients, the levels of albumin were low on admission, never exceeding 2.4 grams per cent. This was true whether or not ascites was present. The levels of globulin were elevated in all cases. In patients No. 1 to 6 (Table II), ascites was present for periods of 1 to 22 months. Cases No. 1 and 2 are remarkable in that 19 and 33 paracenteses respectively were performed and the amounts of fluid removed at each time ranged from 8 to 25 liters. As is shown in the table, ascitic fluid ceased to reaccumulate prior to any increase in the levels of albumin in the plasma. Plasma levels of albumin,

determined at intervals of about 4 weeks, failed to show any significant change until 6 months or more after ascites was absent. In Case 1, for example, followed for a period of 22 months without ascites, no change has occurred in the level of albumin in the plasma, the latest determination in Aug. 1944 being 1.9 grams per cent. Cases 7 and 9 died while still accumulating ascitic fluid. In Case 8, a total of 15 paracenteses have now been performed. Ascites only developed in Case 4 following episodes of severe bleeding. At such times, it was necessary to transfuse the patient and to give 5 per cent glucose infusions. The ascites was so severe as to require paracenteses on 3 occasions. The levels of albumin in the plasma were as low when no ascites was present as during the periods when it occurred.

The levels of plasma albumin in Cases 10 to 14, in whom ascites was not present on admission, were as low as in Cases 1 to 9. The patients, with the exception of case 14, have been followed for periods of 4 to 19 months and the levels of albumin and globulin have been reported at intervals corresponding to those of the previous group. In Cases 10 and 11 who have been followed for the longest period, 14 and 19 months, the albumin levels are now 3.5 and 3.0 grams per cent.

The observations suggested that the level of albumin in the plasma was not the sole deter-

TABLE I
Data on patients

Cases	Age	Sex	Duration of liver symptoms	Jaundice	Ascites	Spider angiomas	Admission findings			
							Bromsulph. retention	Cholesterol		
								Total	Free	Ratio—free/total
	years						per cent	mgm. per cent		per cent
1	46	M	24 months	No	Yes	No	21	124	55	44
2	47	F	7 months	Slight	Yes	Yes	20	169	57	33
3	66	M	2 weeks	No	Yes	No	26	127	41	32
4	39	F	6 months	Yes	At times	Yes	28	130	62	48
5	53	M	18 months	Yes	Yes	Yes	20	193	74	39
6	59	M	6 weeks	No	Yes	No	30	206	78	38
7	57	M	12 months	Yes	Yes	Yes	30	113	69	49
8	54	M	4 months	No	Yes	No	20	145	55	38
9	43	M	6 weeks	Yes	Yes	Yes	35	115	95	35
10	72	M	4 years	No	No	No	28	94	31	34
11	40	M	2 months	No	No	No	23	200	93	49
12	36	F	18 months	Yes	No	Yes	59	115	61	52
13	68	F	3 months	Slight	No	Yes	23	126	45	36
14	53	M	2 months	Slight	No	Yes	35	161	83	59

assay, an amount which represented just under 15 minutes urine output.

Method of Assay. Assay of the antidiuretic activity was performed by the method of Burn (17). The rats used were adult males from the laboratory stock weighing 120 to 240 grams. They had been maintained on a standard Nu Chow diet and water had been given *ad lib.* Food was removed 12 to 18 hours before the assay was performed. Water was allowed up to the time of assay. Series of 4 rats were placed in metabolism cages. Two series were used for each assay and the difference in total rat weight in each series was not greater than 10 per cent. The animals were hydrated to 5 per cent of their body weight with lukewarm tap water, given by means of a gastric tube. Immediately after hydration an intraperitoneal injection of 1 ml. of the solution to be assayed was given to each rat. The urine was collected and the volumes recorded, after the first 30 to 45 minutes, at 15-minute intervals. Fecal contamination was prevented by use of a fine wire mesh and a small amount of glass wool in the neck of the funnel. In a certain number of experiments, determinations of the total chloride excretion in each cage for a 2-hour period were made by the open Carius method of the Volhard titration as applied by Van Slyke (18). Pitressin (Parke Davis), as commercially prepared, was assayed for its antidiuretic activity and for its ability to stimulate the excretion of chlorides. The effect of pitressin added to the urine of normal individuals and dialyzed for 6 to 8 hours was studied in a similar manner.

A total of 200 rats was used in the determinations. Six types of experiment were performed: (1) Assay of known amounts of pitressin (Parke Davis) for antidiuretic activity. (2) Assay of known amounts of pitressin subjected to dialysis for antidiuretic activity. (3) Assay of urine from normal individuals for antidiuretic activity. (4) Assay of urine from patients with cirrhosis of the liver for antidiuretic activity. (5) Establishment of diuretic curves for the rat group when hydrated and given 1 ml. of distilled water intraperitoneally. (6) The total chloride excretion was determined in the urines of the rats in experiments 1, 2, 4, and 5.

RESULTS

In each assay, the diuretic response of 4 hydrated rats was recorded in the form of a graph, plotting the time taken for excretion against the volumes of urine excreted. This latter was expressed as the percentage of the total amount of the water administered. To facilitate comparison, results are expressed as the time in minutes required for the excretion of 25 per cent and 50 per cent of the volume of water administered in the hydration. Each assay was done in duplicate.

Control experiments on diuresis. As good correlation between dose and response has been shown to occur following the injection of known

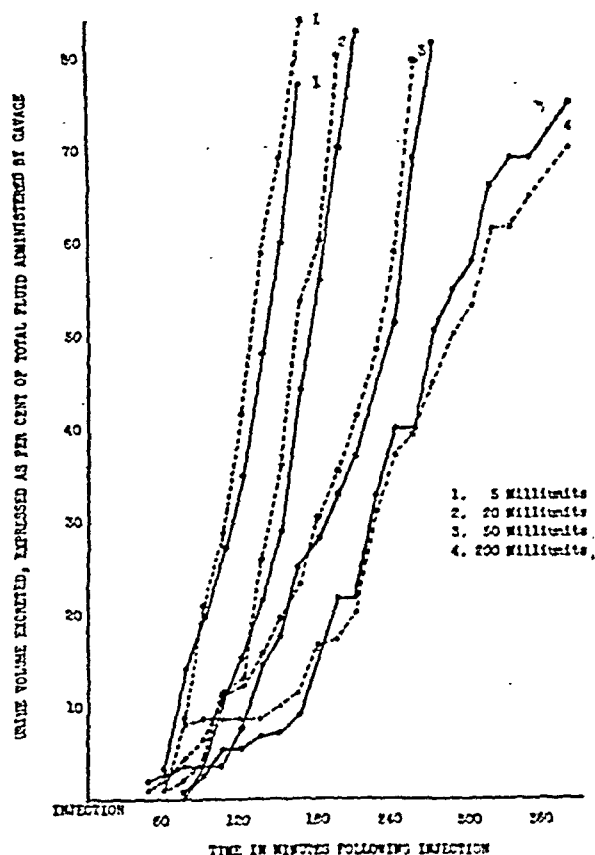


FIG. 1. THE ANTIDIURETIC EFFECT IN HYDRATED RATS OF GRADED DOSES OF PITRESSIN (PARKE DAVIS) GIVEN INTRAPERITONEALLY

amounts of pitressin (16, 28), only a few experiments of this type were performed. In Figure 1 are plotted the results obtained with 5, 20, 50, and 200 milliunits of pitressin given intraperitoneally. The averages of the times required for 25 per cent excretion following injections of the above doses were, respectively, 98, 135, 170, and 210 minutes. The corresponding averages required for 50 per cent excretion were 135, 173, 233, and 278 minutes. The biological response to the doses of pitressin used was greatest between the ranges of 5 to 50 milliunits.

Seven experiments were performed in which known amounts of pitressin were added to about 100 ml. of normal urine and the mixture dialyzed from 7 to 8 hours. The antidiuretic potency of the solution was then assayed. One ml. of this preparation, which had originally contained 20 milliunits of pitressin, was injected intraperitoneally. Six experiments were done in which the

antidiuretic potency of 20 milliuunits of pitressin was assayed without previous dialysis. The anti-diuretic activity of the two solutions was then compared and in all 13 assays the antidiuretic effects of both dialyzed and undialyzed pitressin were found to be of equal magnitude. The results of typical experiments are shown in Figure 2.

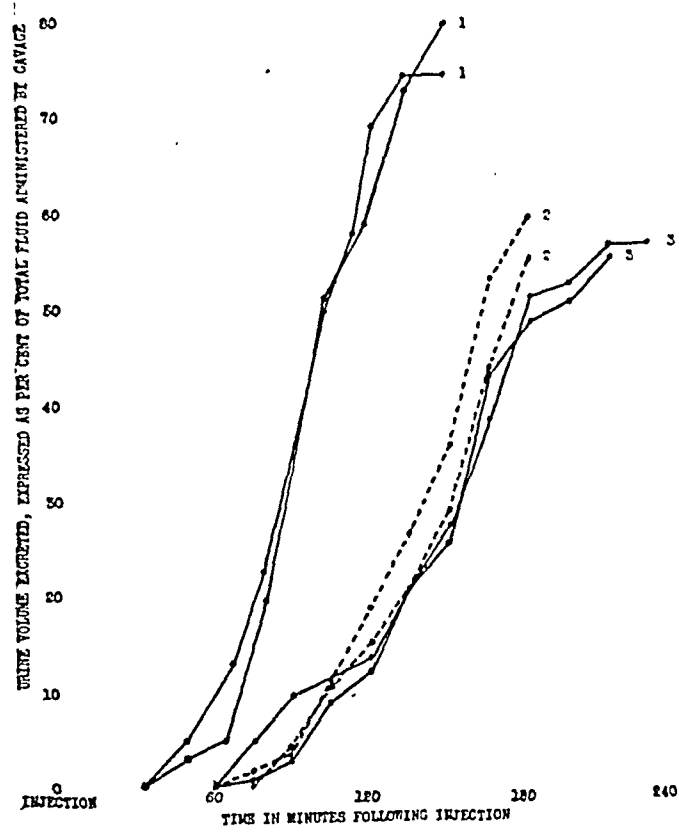


FIG. 2. EFFECT OF DIALYSIS UPON ANTIDIURETIC ACTIVITY OF PITRESSIN (PARKE DAVIS)

1. Hydrated rats given 1 ml. distilled water intraperitoneally. 2. Hydrated rats given 20 milliuunits pitressin (undialyzed) intraperitoneally. 3. Twenty milliuunits of pitressin dialyzed in urine for 7 hours given intraperitoneally to hydrated rats.

There was almost complete superimposition of the excretory curves in the two types of assay. In the experiments in which pitressin was previously dialyzed, the averages of the number of minutes required for 25 per cent and 50 per cent excretion were 135 and 173 minutes, respectively. The corresponding averages for undialyzed pitressin were 135 and 172 minutes.

In order to establish the excretory curve following hydration to 5 per cent of body weight, 12 experiments were performed on groups of 4 rats. One ml. of distilled water was given intra-

TABLE IV
Effect of hydration (5 per cent of body weight) on urine output in rats

Rat groups No.	Time in minutes for excretion of	
	25 per cent	50 per cent
1	75	95
2	75	95
3	60	100
4	60	95
5	65	100
6	75	110
7	85	115
8	85	110
9	85	115
10	85	110
11	80	115
12	75	110
Average	75	106
Range	60 to 85	95 to 115

peritoneally to each animal after hydration to simulate the conditions of the experiments with urine. The results are shown in Table IV. The time required for 25 per cent excretion ranges from 60 to 85 minutes, and for 50 per cent excretion from 95 to 115 minutes, with averages of 75 minutes and 106 minutes, respectively.

Results of assay of human urines. Twenty-four-hour specimens of urine from 4 normal individuals were assayed for their antidiuretic activity. In 1 case, the assay was performed on 2 separate urine samples. The results of the experiments are shown in Table V and indicate that normal urine has a slight antidiuretic activity. The time taken for 25 per cent excretion averaged 97 minutes, and for 50 per cent excretion, the average was 115 minutes. The ranges of the two were 75 to 115 and 125 to 185 minutes, respectively. Assay of antidiuretic activity was performed on 24-hour urine samples from 8 cases with cirrhosis of the liver. In 1 case, two 24-hour samples were assayed. Cases 7, 8, and 9 (Table V) were patients who had massive ascites and considerable peripheral edema at the time the urine was assayed. Cases 1, 2, and 4 were patients in whom massive ascites and edema had been present for a considerable time but in whom there was no ascites demonstrable at the time of the assay. In these cases, ascitic fluid had not accumulated for periods of 3 to 16 months prior to the assay. Cases 10 and 14 were patients with severe hepatic cirrhosis in whom ascites and edema had never been present to any demonstrable extent. In Cases 7, 8, and 9, the time required for 25 per cent excretion ranged from 175 to 215 minutes.

TABLE V

Effects on urine output in hydrated rats of the intraperitoneal injection of the extract of urines of normal and cirrhotic subjects

	Cases with cirrhosis of the liver								Normal subjects					
	Case no.	Sex	Sp. gr.	24-hour urine vol.	Degree of ascites	Periph. edema	Time for excretion of		Case no.	Sex	Sp. gr.	24-hour urine vol.	Time for excretion of	
							25 per cent	50 per cent					25 per cent	50 per cent
Patients with massive ascites	8-A	M	1.020	ml. 1000	4+	2+	minutes 195 360 215 370		R	M	1.010	ml. 1350	minutes 95 130 110 135	
	8-B	M	1.020	585	4+	2+	190 320 175 305		D	F	1.010	1600	75	150
	9	M	1.020	800	3+	2+	195 300 190 330		S	M	1.025	1100	90 95	180 185
	7	M	1.030	410	3+	2+	180 225 175 245		G-1	M	1.015	1080	85 105	120 135
Patients with no clin. ascites at time of urine assay	2	F	1.015	1600	0	0	150 200 145 205		G-2	M	1.014	1200	100 115	125 145
	4	F	1.015	1420	0	0	150 215 155 205		Average 97 Range 75 to 115				145 120 to 185	
	1	M	1.010	1650	0	0	130 160 150 190							
Ascites never present	14	M	1.014	1500	0	0	125 160 110 165							
	10	M	1.014	1320	0	0	75 105 85 120							

In Cases 1, 2, and 4, excretion of 25 per cent of administered water occurred from 130 to 155 minutes, while in Cases 10 and 14, the time required was 75 to 125 minutes. The corresponding values for 50 per cent excretion were 225 to 370 minutes, 160 to 215 minutes, and 105 to 165 minutes, respectively.

It is clear that the urine of patients with cirrhosis of the liver and ascites had a greater antidiuretic effect when injected into hydrated rats than did the urine from patients without ascites. Moreover, the magnitude of the effect seemed to parallel the degree of ascites. As can be seen from the results in Table V, the antidiuretic activity of the urine of patients 10 and 14, in whom ascites had never been present clinically, fell within the range of the antidiuretic activity of the urine of normal persons.

Observations on chloride excretion. In order to examine the effects upon chloride excretion of the extract of urine from patients with cirrho-

sis of the liver, the total chloride excretion was determined in groups of 4 rats hydrated to 5 per cent of their body weight. The results are shown in Table VI. The total chloride is expressed in micro-equivalents per 100 grams of rat weight, excreted in the 2-hour period following the intraperitoneal injection. The total chloride excretion in Figure 3 is expressed in micro-equivalents per 100 grams of rat weight for a 2-hour period and is plotted against the antidiuretic effect obtained with the substance injected. The urine output in the 2-hour period was measured and that volume, expressed as a percentage of the total amount of water administered by gavage, is used to indicate the antidiuretic response.

Five experiments were performed in which 1 ml. of distilled water was injected intraperitoneally into hydrated rats. The total chloride excretion per 100 grams of rat weight in the ensuing 2-hour period ranged from 238 to 350 micro-equivalents, with an average of 273 micro-

equivalents. In 6 experiments in which hydrated rats were given 20 milliunits of pitressin (Parke Davis) in a volume of 1 ml. of water, the chloride excretion per 100 grams of rat weight in the same period ranged from 51.3 to 67.6 micro-equivalents, the average being 60.2 micro-equivalents. Commercial pitressin was added to the urine of a normal individual and dialyzed for 6 to 8 hours. Six experiments were performed in which 1 ml. of this solution, which had originally contained 20 milliunits of pitressin, was assayed for its antidiuretic activity and its ability to stimulate the excretion of chlorides. As previously mentioned, there was no loss in antidiuretic potency. When injected intraperitoneally into hydrated rats, the total chloride excretion per 100 grams of rat weight per 2-hour period ranged from 20.1 to 34.4 micro-equivalents. The average chloride excretion in these experiments was 27.6 micro-equivalents. Six experiments were performed in which 1 ml. of a urine extract from a patient with cirrhosis of the liver was given intraperitoneally to hydrated rats. The chloride excretion per 100 grams of rat weight per 2-hour

TABLE VI

Observations on chloride excretion

Total chloride excreted per 100 grams rat weight in 2-hour period after hydration to 5 per cent of body weight, and injected as indicated.

Chlorides are expressed in micro-equivalents

Exp. no.	1 ml. distilled water injected intra-peritoneally	20 milliunits of pitressin (Parke-Davis) injected intra-peritoneally	20 milliunits of pitressin-dialyzed injected intra-peritoneally	1 ml. urine extract from patients with cirrhosis of liver and ascites, injected intra-peritoneally
1	27.0 27.4	67.6 67.2	34.1 34.4	23.5 23.1
2	25.6 26.7	51.8 51.3	29.6 30.1	21.9 21.2
3	23.8 24.3	63.1 63.5	22.5 22.0	19.0 19.0
4	34.5 35.0	65.7 65.1	20.1 20.6	21.9 21.2
5	23.9 25.1	54.5 53.5	24.4 24.7	12.6 13.0
6		60.3 59.1	34.2 34.3	15.4 15.8
Range	23.8 to 35.0	51.3 to 67.6	20.1 to 34.4	12.6 to 23.5
Average	27.3	60.2	27.6	19.2

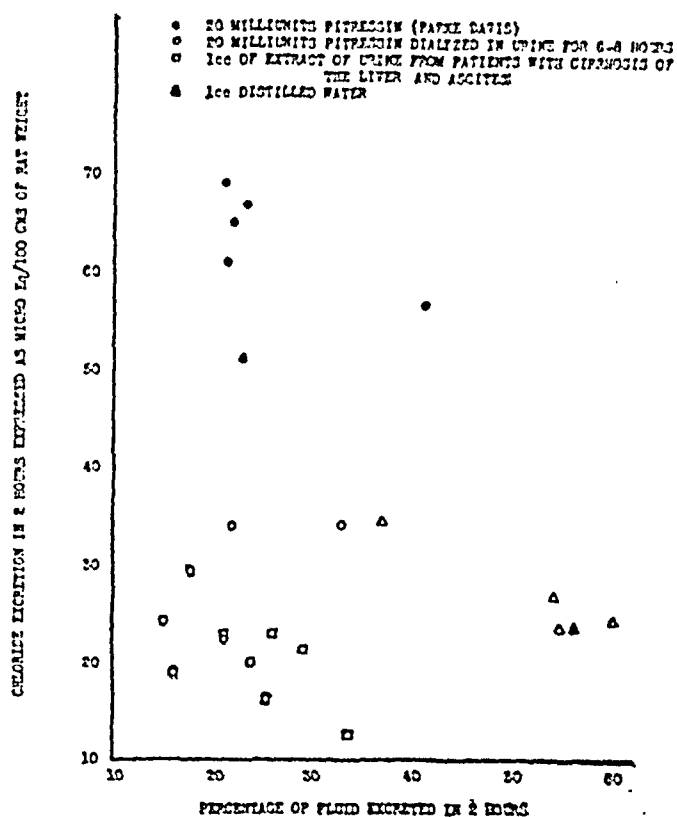


FIG. 3. WATER AND CHLORIDE EXCRETION IN HYDRATED RATS FOLLOWING THE INTRAPERITONEAL INJECTION OF COMMERCIAL PITRESSIN, DIALYZED PITRESSIN, THE EXTRACT OF URINE FROM PATIENTS WITH CIRRHOSIS, AND DISTILLED WATER

period ranged from 12.6 to 23.5 with an average excretion of 19.2 micro-equivalents. The results indicate that the antidiuretic factor present in the urine of patients with cirrhosis of the liver does not possess the ability to stimulate the excretion of chlorides. They confirm the chloruretic activity of undialyzed pitressin (19 to 22) and show that this property is lost by dialysis.

DISCUSSION

Our observations on the plasma levels of albumin in patients with cirrhosis of the liver, both with and without ascites, suggest that ascites in such patients is not determined solely by the level of albumin in the plasma or by the oncotic pressure thereby exerted. Moreover, it would seem that far too much emphasis has been placed on the critical level of albumin in the plasma as a determining factor in the production of ascites. Not only do the data that we have presented fail to support this contention but there are several reports in the literature which agree with this

idea. In 1939, Butt, Snell and Keys (23) reported the oncotic pressure in patients with cirrhosis of the liver and in patients with obstructive jaundice. Only 2 of their patients with obstructive jaundice had ascites, while ascites was present in all of the patients with cirrhosis. The oncotic pressures, however, were as low in the patients with obstructive jaundice as in the patients with cirrhosis and ascites.

In the patients we have observed in whom ascites was controlled, the plasma levels of albumin have remained well below the normal value for at least 6 months. Until more cases have been studied one cannot know whether or not the plasma levels of albumin will ever return to and remain at the accepted lower level of normal, 3.8 grams per cent (24). It is equally significant that in the patients in whom ascites was never present, the plasma levels of albumin were all below the normal level.

The observation that there is a decrease in urine output in patients with cirrhosis of the liver is not new. In 1901, Gilbert and Lereboullet (13) reported that the output of urine was decreased in such patients. Pick (25), in 1929, stated that in certain cases of nephrosis there was an increased diuresis following liver extract therapy. He discussed the question of a "liver hormone" in relation to water metabolism and cited the experimental work of Glaubach and Molitor on the effect of an injection of liver extract on water diuresis in a dog with kidney damage. Ratnoff and Patek (26) also mention the water retention in cirrhosis of the liver but consider that the "lowering of serum albumin and therefore of oncotic pressure is ample explanation for water retention in most cases of cirrhosis of the liver." They agree, however, that there may be other factors involved.

The nature of the antidiuretic effect which we have observed with urines from patients with cirrhosis of the liver cannot be defined at this time. That the effect may be due to a substance of pituitary origin should be considered. The method that was used for its extraction from the urine was that described (15, 27, 28) for the extraction of the antidiuretic hormone of the pituitary. All of these investigators found that with this method, the substance could not be extracted from the urine of animals unless the posterior pituitary or

the hypothalamic-hypophyseal system was intact. Furthermore, with this method an antidiuretic substance was recovered (14) from the urine following the injection of pitressin into normal individuals, and the recovered substance exerted an antidiuretic effect equal to that of the amount of pitressin injected. It has been argued (29) that the substance extracted from urine is not pituitary in origin because it does not cause, when injected, an increased excretion of chlorides. This statement is contradicted by our experiments which show that the chloruretic activity of commercially prepared pitressin is lost by dialysis although the antidiuretic potency remains unchanged. If the substance is pituitary in origin, it may be that the reason for finding it in increased concentrations in the urine of patients with cirrhosis of the liver and ascites is due to the fact that the damaged liver has lost the capacity to inactivate this hormone. There is evidence that the liver does inactivate some of the hormones and it has been reported that a suspension of liver may inactivate the antidiuretic hormone of the pituitary (30).

Another factor which may enter into the increase in concentration of the antidiuretic substance in the patients with ascites and cirrhosis is dehydration. Ham and Landis (29) reported that, in 1 female subject "dehydrated to a moderate degree," there was a moderate amount of antidiuretic substance excreted in the urine. In one of our normal male subjects, fluids were restricted so that the total urine output for 24 hours was 930 ml. and the specific gravity was 1.032. The antidiuretic effect of this urine was in the upper limits of normal; *e.g.*, the time in minutes required for the excretion of 25 per cent of the ingested water in the rats averaged 123 minutes whereas the upper limit of normal in the other 4 subjects was 115 minutes. Fifty per cent of the ingested water was excreted in 180 minutes by the rats receiving the antidiuretic extract from this patient's urine.

Although it is indubitably true that the plasma levels of albumin are low in patients with cirrhosis of the liver, the evidence that this is the determining factor in fluid retention is open to question. The very fact that the albumin level is low, whether or not ascites is present in patients, would in itself contradict the hypothesis. Our

observations have shown that there is some correlation between the degree of ascites in patients with cirrhosis and the antidiuretic activity of the urine. Granted, that there is a certain amount of dehydration present in the patients with ascites, the possibility still exists that fluid retention in such patients may be influenced by some antidiuretic factor which is effective in suppressing urine output. The exact nature of this substance is not known but it is possible that it may have its origin in the posterior pituitary. The fact that the antidiuretic effect is not equally present in all patients with cirrhosis of the liver, but is marked in the patients with ascites, suggests that, in spite of severe liver damage, all functions of the liver are not equally impaired. In the patients in whom ascites has not occurred, it is probable that the capacity of the liver to detoxify certain substances is still intact. The cessation of the accumulation of ascitic fluid in 6 of the patients treated with the high carbohydrate, high protein diet and with liver extract, confirms the observations of Patek and Post (31) that this combined form of therapy is indicated in the treatment of patients with cirrhosis of the liver. These investigators administered liver extract, 5 ml. intramuscularly, twice weekly along with a high protein diet.

SUMMARY

Observations are reported on the plasma levels of albumin and globulin in patients with cirrhosis of the liver, with and without ascites. The plasma levels of albumin were low in both groups. Following treatment with diet and intravenous liver extract, reaccumulation of ascitic fluid ceased in 6 patients and this occurred before there was any increase in the levels of albumin in the plasma. Determinations of the plasma at monthly intervals in the patients showed no significant increase in the level of albumin for as long as 6 months after the ascites was absent.

The urine of the patients with and without ascites and of normal subjects was assayed for its antidiuretic activity. It was found that the urine of patients with ascites when injected into hydrated rats delayed the excretion of urine. The urine from patients in whom ascites was never present possessed an antidiuretic effect similar to that obtained with the urine from normal subjects.

The urine of patients in whom ascites had been controlled prior to assay had an antidiuretic effect greater than the urine of normal subjects but less than that obtained with urine from patients with ascites. The nature of the antidiuretic substance obtained from the urine has not been ascertained. Based on the methods of extraction and on the work of other investigators, it is suggested that the antidiuretic substance may have its origin in the posterior pituitary.

Pitressin as commercially prepared was found to exert its full antidiuretic activity after dialysis. This procedure, however, resulted in a loss of the chloruretic effect of the hormone.

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THE CARDIAC OUTPUT IN MALE SUBJECTS AS MEASURED BY THE TECHNIQUE OF RIGHT ATRIAL CATHETERIZATION. NORMAL VALUES WITH OBSERVATIONS ON THE EFFECT OF ANXIETY AND TILTING¹

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The determination of the cardiac output in man has usually been done by indirect methods. The demonstration (1) that mixed venous blood could be obtained by introducing a catheter into the right atrium and the later data (2), showing that this technique is simple and safe, have provided a new method for quantitative studies of the circulation in man. The purpose of this paper is to report the data obtained in the study of normal resting subjects by the catheter technique. Some of these persons were relaxed and quiet; others were nervous and apprehensive.

METHODS

Physicians, medical students, hospital patients, and paid volunteers served as subjects. They had eaten no food since the evening meal on the night previously and they came to the laboratory at 7:30 a.m. After resting one-half hour, the procedure was begun. Thirty to 90 minutes later, quantitative studies were made.

The right atrium was catheterized via the median antecubital vein by the technique described by Cournand *et al.* (2 to 4). A slow drip of physiological saline solution was maintained through the catheter during the study. The necessity has been stressed (4) for having the tip of the catheter in the region of the tricuspid valve in order to obtain blood in which the streams from the superior and inferior cavae are fully mixed. Even with this precaution, a sample of mixed venous blood may not be obtained. In 3 subjects, blood from the atrium in the region of the tricuspid valve was found to have a very low oxygen content. Samples taken from the right ventricle or other parts of the atrium led to the conclusion that the catheter had entered the coronary sinus, or an aberrant hepatic vein emptying directly into the atrium.

An inlying needle was placed in the femoral artery, which had previously been well novocainized. Oxygen consumption was measured by collecting expired air for

2- or 3-minute periods in a Douglas bag and analyzing its oxygen and carbon dioxide content by the method of Haldane. It was found necessary to bring in air from the outside of the building as the carbon dioxide content of the room air increased appreciably during the experiment. The metabolic rate was recorded as the percentage of deviation from the expected basal metabolism. The oxygen content of the mixed venous and arterial bloods was determined by the method of Van Slyke and Neill (5). The hemoglobin concentration of the blood was measured by diluting the blood with ammonia water and determining the intensity of the color by a photoelectric colorimeter. In the few instances where an arterial puncture was not done, blood from the atrium was saturated with oxygen and its oxygen capacity determined directly. The arterial oxygen content of the blood was then calculated on the assumption that the hemoglobin in the arterial blood was 95 per cent saturated with oxygen. It was not possible to calculate the oxygen carrying capacity of the blood from the concentration of the hemoglobin. Varying amounts of inactive hemoglobin were found in the blood. Similar observations have been reported by other investigators (6, 7).

The arterial pressure was recorded optically from the femoral artery by the method of Hamilton (8). The mean arterial pressure was measured by planimetric integration of the area beneath the tracing. The peripheral resistance was recorded in absolute units and calculated by the formula (2):

$$R = \frac{P_m \text{ (mean pressure in mm. Hg)} \times 1332}{C.O. \text{ (cardiac output in ml. per sec.)}}$$

The mean atrial pressure was measured by a manometer filled with physiological saline solution, a point 5 cm. below the fourth costo-chondral junction being taken as the center of the right atrium. The ventilation was expressed in liters of air per minute per sq. meter at 37° and the prevailing barometric pressure (9).

Three of these subjects had an intravenous injection of 2 ml. of a 20 per cent solution of para-amino hippurate from 15 to 20 minutes before the cardiac output was measured. The disappearance rate of this substance was being determined in an associated study and, as this substance was found to have no effect on the circulation, the data on these patients have been included along with those of other normal subjects.

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Emory University School of Medicine.

RESULTS

Eleven relatively complete sets of observations were made on 7 medical students and 2 physicians (Table I). These subjects were familiar with the techniques used and had been present when the procedures were carried out on other subjects. In 5 of these experiments, the measurements were made before the removal of blood for an associated study. The subjects had served as donors previously and were not apprehensive about the loss of blood. Eight of the subjects appeared relaxed. The ninth was obviously tense. In the 10 sets of observations on normal, relaxed subjects, the ventilation rate varied from 2.4 to 3.5, the oxygen consumption per sq. m. from 103 to 133 ml. per minute. The arteriovenous oxygen difference ranged from 3.1 to 5.0 and the cardiac output from 4.9 to 7.7. The cardiac index varied from 2.4 to 3.9 and the mean femoral pressure from 71 to 97 mm. of Hg. The pressure in the right atrium ranged from 15 to 75 mm. of water, the peripheral resistance from 822 to 1580 units. Observations were made on 2 different days on one of these subjects. On the first occasion, the metabolic rate deviation from the expected normal was minus 25, on the second, minus 13. Two determinations of the oxygen consumption were done with each experiment. The first set of observations may represent a technical error, but we have not been able to detect it. In the tense subject, WB, the heart rate was rapid, the oxygen consumption was slightly higher than in the others, the cardiac output was greatly increased and the peripheral resistance was extremely low. Five months later, the observations were repeated. The subject was more relaxed, the pulse rate had decreased 18 beats per minute, the cardiac output had fallen to one-half its former level, and the peripheral resistance had risen 400 units. The metabolic rate had decreased from plus 6 to minus 12.

Similar studies were made on 10 colored male patients from the wards of Grady Hospital. Four of the subjects were convalescing from an herniorrhaphy, one had asymptomatic neurosyphilis, one diffuse neurofibromatosis, and one submental tuberculous lymphadenitis without any systemic evidence of infection. Three patients were convalescing from acute illness, one from typhus

fever, one from infectious hepatitis, and one from lymphocytic choriomeningitis. As a rule, these patients showed somewhat higher ventilation rate and oxygen consumption than did the medical students and physicians. The average oxygen consumption per minute per sq. m. was 139 ml., as compared to 121 ml. for the students and physicians. It was much more difficult to judge whether these patients were relaxed. In one subject, the metabolic rate was plus 16. In a second, the pulse rate was 84 beats per minute. Excluding these 2 subjects, the cardiac index varied from 2.3 to 4.1 and the atrial pressure from 0 to plus 85 mm. of water. The cardiac index in the students and physicians who appeared relaxed averaged 3.2. This is not significantly different from the average figure of 3.6 found in the colored patients.

Three negro males were studied before and after venesection. These subjects came into the hospital the morning of the experiment. They had never given blood and were naturally somewhat apprehensive. The oxygen consumption in these patients was definitely above the level of the controls and the cardiac output was elevated. The first subject, WP, was of particular interest because observations were made at 3 different times. By the third experiment, he was well relaxed. In the first 2 experiments, the oxygen consumption was 14 and 8 per cent higher than in the third one, while the cardiac output was 47 and 65 per cent higher than in the third set of observations.

Observations on the effect of gravity on the cardiac output were made in 5 normal subjects and one patient with hyperthyroidism. After the cardiac output was measured with the subject in the recumbent position, the table was tilted to an angle of approximately 70° from the horizontal, the feet resting against an upright foot support. The arms hung loosely at the thighs and the persons were relaxed except for the use of muscles necessary to the maintenance of the ventilatory posture. Care was taken not to move the legs or to tighten and relax the leg muscles alternately. The position of the catheter was checked by fluoroscopy and after 5 to 8 minutes of standing the cardiac output was measured. The results are given in Table II. In each instance,

TABLE II

Effect of tilting to 70° on the oxygen consumption, arteriovenous oxygen difference, and cardiac index of 5 normal subjects and one patient (P W) with hyperthyroidism

Subject	Sur- face area	Horizontal			Tilted to 70°		
		O ₂ con- sump.	A-V O ₂ differ- ence	Car- diac index	O ₂ con- sump.	A-V O ₂ differ- ence	Car- diac index
	square meters	ml. per min. per sq. meter	vol- umes per cent	liters per min. per sq. meter	ml. per min. per sq. meter	vol- umes per cent	liters per min. per sq. meter
W F	1.85	114	4.3	2.7	129	5.6	2.3
R G	1.98	129	4.2	3.1	130	5.3	2.5
W M	1.90	120	3.1	3.9	131	6.2	2.1
M S	1.87	130	5.0	2.6	124	6.3	2.0
W B	1.94	122	3.1	3.9	140	4.4	3.2
P W	1.49	164	3.3	5.0	170	4.4	3.9
Average		130	3.7	3.5	139	5.4	2.7

the arteriovenous oxygen difference increased and the cardiac output decreased. In the semi-erect position, the increase in arteriovenous oxygen difference was somewhat more striking than the fall in cardiac output, because the metabolic rate was usually higher with the patient in the semi-erect than in the horizontal position. In one subject, 3 specimens of blood were taken from different areas in the right atrium to be certain that the lowering of the oxygen content of the atrial blood was not the result of improper mixing of the blood from the superior and inferior venae cavae. The values found for the oxygen content were similar in the 3 specimens.

COMMENT

Determination of the cardiac output utilizing mixed venous blood from the right atrium has opened a wide field for clinical investigation. This technique, however, has many sources of error so that small changes are difficult to interpret. The method is more accurate when the arteriovenous oxygen difference is large. When the oxygen consumption remains constant, the relationship between the cardiac output and the arteriovenous oxygen difference is a logarithmic one. Thus, with an arteriovenous oxygen difference of 8, an increase of one volume per cent will cause a decrease in cardiac output of only 12 per cent. With an arteriovenous difference of 2,

an increase of one volume per cent will cause a 33 per cent decrease in output (Figure 1).

Other investigators (2) have taken repeated samples within a few minutes' time in a series of 11 subjects with the subject in an apparently steady state. The mixed venous oxygen in successive samples varied as much as 0.5 volume per cent. With an arteriovenous oxygen difference of 4, this much variation would cause a 12.5 per cent variation in cardiac output. These same authors have emphasized the care that must be taken in locating the tip of the catheter close to the tricuspid valve. Even then variations in oxygen content because of admixture of blood from the coronary sinus cannot be controlled. The collection of 2-minute samples of air for determining oxygen consumption introduces an error which probably varies considerably from subject to subject. In any given subject, it is difficult to be certain that he is relaxed and that an increase or decrease in cardiac output attributed to

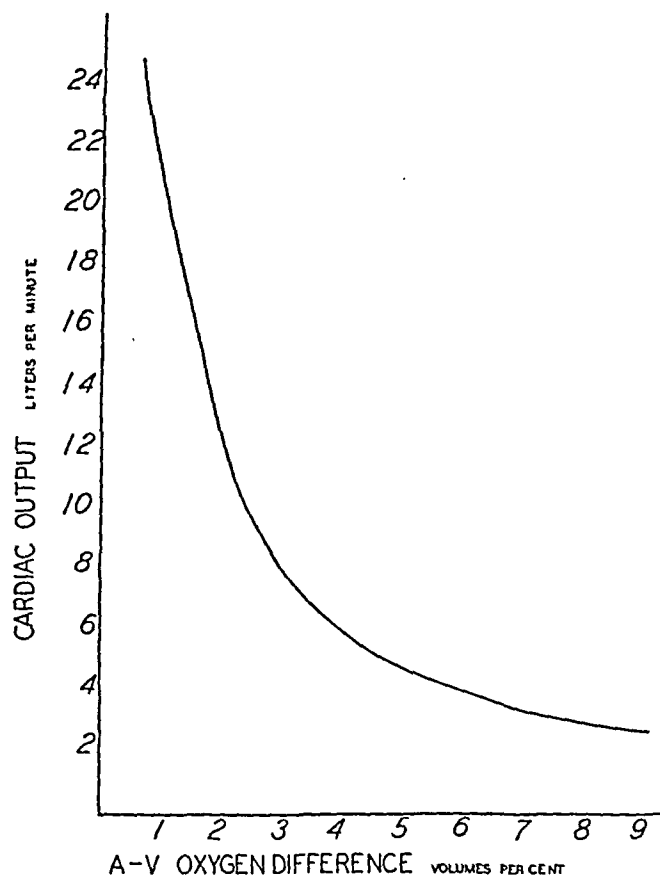


FIG. 1. THE RELATIONSHIP BETWEEN THE CARDIAC OUTPUT AND THE ARTERIOVENOUS OXYGEN DIFFERENCE, ASSUMING THAT THE OXYGEN CONSUMPTION REMAINS CONSTANT

an experimental procedure is not merely the result of variation in the degree of relaxation.

These data demonstrate that, in normal adult males, the cardiac output at rest varies greatly, ranging from 4.2 to 14.8 liters per minute. The output per minute per sq. m. of body surface (cardiac index) ranged from 2.3 to 7.7. It was obvious that certain of the subjects were not well relaxed and that, therefore, the circulation was hyperactive. The data from these subjects should not be used in determining the normal range of the various functions of the circulation in relaxed subjects under basal conditions. It was arbitrarily decided to exclude from the normal relaxed group subjects in whom the metabolic rate was over plus 10 and in whom the heart rate was over 82 beats per minute. From the analysis of the data on 19 studies in 18 normal subjects, the following range of normal values is obtained: Ventilation rate from 2.4 to 5.6 with an average of 3.4; oxygen consumption per sq. m. per minute from 103 to 146 with an average of 128 ml.; arteriovenous oxygen difference from 3.1 to 6.1 with an average of 4 volumes per cent; cardiac output per minute per sq. m. of body surface from 2.3 to 4.1 with an average of 3.3; mean femoral pressure (data on 13 subjects) from 71 to 98 with an average of 85 mm. Hg; atrial pressure from 0 to 85 with an average of 31 mm. of water; peripheral resistance from 820 to 1750 with an average of 1160 units.

Cournand *et al.* have reported observations on 15 normal subjects (2). The data given there are recorded in the next to last line of Table I. Our subjects had lower pulmonary ventilation, a lower oxygen consumption, and a somewhat smaller arteriovenous oxygen difference. A similar range of cardiac index is noted in the two studies. The average atrial pressure in the two series was not significantly different. The values for the arteriovenous oxygen differences recorded here are in general agreement with those found in normal subjects by McMichael and Sharpey-Shafer (10).

The values for the cardiac output as measured by the catheter method are greater than those obtained by the acetylene technique. This has been a consistent finding in each of the reported series (2, 10, 11).

A decrease in cardiac output occurred when

the body was tilted to an angle of 70° from the horizontal. The subjects in these experiments were partially supported by leaning against the table and therefore the experiments are not directly comparable to those in which the subject has been standing relaxed, but without support. Grollman (11), using the acetylene method, found that the cardiac output was unchanged when the patient stood in a relaxed position. The arteriovenous difference increased when the patient stood, but the oxygen consumption also increased so that the cardiac output remained unchanged. One author (12), using the acetylene method, found that the cardiac output with the subject standing in a relaxed position averaged 9 per cent less than with the subject in the horizontal position. More recently, the same author (10), using the catheter technique, found that the cardiac output in the relaxed standing position was 25 per cent lower than in the recumbent position. Others (13), using the ballistocardiograph to measure the cardiac output, found no consistent change when observations were made with subjects in the recumbent and relaxed standing positions. Our data are not comparable because our subjects were partially supported and not standing erect.

Seven sets of observations on 5 subjects were not included in the data on normal basal resting subjects. In 5 out of the 7 experiments, the resting pulse rate exceeded 82 beats per minute. In 5 of the 7, the metabolism was over plus 10. The average figures for the various aspects of the circulation in this group of subjects with some evidence of anxiety are given in the last line of Table I. The average A-V oxygen difference was 3.1, the average cardiac index 5.5, and the average peripheral resistance 827. It is of interest to note that the average oxygen consumption with anxiety was 23 per cent greater than in the more relaxed subjects, while the cardiac output was increased 66 per cent above that found in the relaxed subjects. The validity of this observation is supported by the data on the 2 subject in whom repeat determinations were made at a subsequent date. In each subject, anxiety produced a much greater rise in cardiac output than in metabolism.

TABLE I
Observations on 22 normal male subjects in the horizontal position
No food for 12 hours before experiment

Subject	Age	Weight	Surface area	Ventilation	Oxygen consumption	Metabolic rate— deviation from normal basal	Arterial O ₂ content	Mixed venous O ₂ content	A-V oxygen difference	Cardiac output	Cardiac index	Femoral arterial pressure			Pulse rate	Atrial pressure	Peripheral resistance
												Systolic	Diastolic	Mean			
	years	kgm.	square meters	liters per min. per sq. m.	ml. per min. per sq. m.	per cent	volumes per cent			liters per min.	liters per min. per sq. m.	mm. Hg			beats per min.	mm. H ₂ O	abso- lute units
Group One—Normal subjects—Students and physicians																	
ES	35	82.6	2.04	3.1	109	-18	18.3	13.7	4.6	4.9	2.4	136	73	91	61	55	1515
WM	23	72.6	1.90	2.4	103	-25	19.4	16.2	3.2	6.1	3.2	116	62	81	60	45	1055
WM	23	72.6	1.90	2.6	120	-13	19.0	15.9	3.1	7.4	3.9	†110	80		58	35	
WLM	22	82.1	2.04	2.7	121	-12	21.2	16.5	4.7	5.3	2.6	112	55	71	60	35	1085
CG	22	68.9	1.91	3.5	131	-5	18.5	14.7	3.8	6.5	3.4	141	75	96	72	35	1160
WS*	25	81.7	2.04	3.1	133	-2	19.2	15.7	3.5	7.7	3.8	117	63	79	79	15	822
WF	23	68.0	1.85	2.5	114	-12	19.4	15.1	4.3	4.9	2.7	†110	60		69	55	
MS	22	72.6	1.87	2.9	130	-6	20.6	15.6	5.0	4.9	2.6	136	73	97	80	75	1580
RG*	23	78.5	1.98	2.6	129	-7	18.6	14.4	4.2	6.1	3.1	†120	76		69		
WB†	23	75.8	1.93	2.6	146	+6	20.2	18.3	1.9	14.8	7.7	128	66	88	100	0	475
WB	23	76.2	1.94	2.5	122	-12	19.5	16.4	3.1	7.6	3.9	113	66	85	82	35	880
Group Two—Normal subjects—Hospital patients without clinical evidence of infection																	
LM*	30	73.9	1.84	4.0	138	+3	20.7	14.6	6.1	4.2	2.3	129	71	92	72	0	1750
BJ†	25	82.1	1.99	3.3	140	+4	19.6	14.9	4.7	5.9	3.0	122	65	82	84	-5	1115
HB†	26	78.0	1.99	3.5	159	+16	18.3	15.1	3.2	9.9	5.0	136	79	100	84	40	807
JC	16	56.2	1.69	4.3	130	-14	17.9	13.8	4.1	5.4	3.2	120	63	81	65	15	1200
PM	17	69.6	1.78	3.8	143	-5	19.6	15.6	4.0	6.4	3.6	120	64	84	73	25	1045
CL	33	64.9	1.77	3.7	135	0	19.7	15.9	3.8	6.3	3.6				60	25	
SL	27	66.2	1.76	3.2	129	-6	16.8	13.5	3.3	6.8	3.9				64	85	
Group Three—Normal subjects—Hospital patients convalescing from acute infectious disease																	
GH	39	77.1	1.93	5.6	130	-5	16.0	11.9	4.1	6.3	3.3	124	68	87	68	10	1100
JF	27	58.5	1.63	4.0	146	+6	16.5	12.5	4.0	6.0	3.7	114	60	73	56	60	972
YW	25	67.1	1.82	4.1	138	+1	19.2	15.8	3.4	7.4	4.1	110	72	76	75	0	823
Group Four—Normal subjects before venesection																	
WP†	32	68.0	1.74	4.8	162	+21	15.6	12.4	3.2	8.8	5.1	126	74	92	75	50	833
WP†	32	68.0	1.74	3.3	153	+12	14.5	11.8	2.7	9.9	5.7	141	80	100	80	45	808
WP	32	68.0	1.74	4.9	142	+5	16.0	11.9	4.1	6.0	3.4	143	75	98	68	50	1305
JT†	31	70.8	1.84	4.6	181	+32	18.5	15.6	2.9	11.5	6.2	165	87	115	112	40	786
IR†	22	72.6	1.84	4.6	160	+16	18.4	15.5	2.9	10.2	5.5	167	94	123	88	30	964
Average values obtained from 19 experiments on 18 normal males in the basal state																	
				3.4	128	-7			4.0		3.3	124	67	85	68	31	1160
Average values for 15 normal subjects reported by Cournand <i>et al.</i> (2)																	
				5.1	154				4.5		3.4			97		33	1290
Average values obtained from 7 experiments on 5 resting subjects with evidence of anxiety																	
				3.8	157	+15			3.1		5.5	141	78	100		29	827

* Received injection of sodium para-amino hippurate.

† Not included in group of relaxed subjects because pulse rate was over 82 or metabolic rate over plus 10. The average values for these subjects with evidence of increased metabolism and hyperactive circulation are listed in the last line of the table.

‡ Pressure measured in arm by auscultatory method.

CONCLUSIONS

1. Studies on the circulation were performed in 22 normal subjects in the basal state. Samples of mixed venous blood and measurements of the atrial pressure were obtained by inserting a catheter through the antecubital vein into the right atrium. The femoral arterial pressure was recorded optically by the method of Hamilton.

2. Nineteen experiments were carried out on 18 subjects in whom the pulse rate was not above 82 beats per minute and in whom the metabolic rate did not exceed plus 10. The arteriovenous oxygen difference varied from 3.1 to 6.1 with an average of 4 volumes per cent. The cardiac index (liters per min. per sq. meter) ranged from 2.3 to 4.1 with an average of 3.3. The atrial pressure ranged from 0 to 85 with an average of 31 mm. of water.

3. Seven experiments were performed on 5 normal subjects in whom either the pulse rate exceeded 82 or the metabolic rate plus 10. These findings were interpreted as evidence of anxiety. The average arteriovenous oxygen difference was 3.1 volumes per cent. The cardiac index averaged 5.5 and the atrial pressure 29 mm. of water. The increase in cardiac output was out of proportion to the increase in oxygen consumption. The rise in cardiac output occurred without any measurable change in atrial pressure.

4. When the subject was tilted to an angle of 70°, the arteriovenous oxygen difference rose and the cardiac output decreased. The average decrease in the cardiac index when the subject was tilted amounted to 23 per cent.

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THE CARDIAC OUTPUT IN PATIENTS WITH CHRONIC ANEMIA AS MEASURED BY THE TECHNIQUE OF RIGHT ATRIAL CATHETERIZATION¹

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Many patients with circulatory failure either from hemorrhage or heart failure have anemia. In these patients, the cardiac output may be within normal limits and yet the circulation may be inadequate. The question immediately arises as to what is the effect of anemia on the requirements of the tissues of the body for blood. Before it can be determined whether a given patient with anemia has an adequate circulation, it is necessary to know how anemia *per se* affects the cardiovascular system and what level of cardiac output should be expected for a given hemoglobin concentration. Studies on the circulation were, therefore, carried out on patients with chronic anemia in whom the picture was not complicated by shock and by the marked decrease in total blood volume which are so often present in the acute anemia produced by hemorrhage.

METHODS

The cardiac output was measured by the utilization of the direct Fick principle. Samples of mixed venous blood from the right atrium and measurements of atrial pressure were obtained from an inlying atrial catheter, introduced into the venous system through the antecubital vein (1, 2). The oxygen consumption was determined by the analysis of a 2-minute sample of expired air, using the method of Haldane. The metabolic rate was recorded as the percentage of deviation from the expected basal metabolism. The oxygen content of the blood was measured by the method of Van Slyke and Neill (3). A point 5 cm. below the 4th right costochondral junction was used as the reference point in reading the atrial pressure. The arterial pressure in the femoral artery was recorded optically by the method of Hamilton (4). The mean arterial pressure was measured by planimetric integration of the area beneath the tracing. The peripheral resistance was calculated by

the formula (2):

$$R = \frac{P_m \text{ (mean pressure in mm. Hg)} \times 1332}{C.O. \text{ (cardiac output in ml. per sec.)}} \quad \checkmark$$

The ventilation was calculated as liters of air ventilated per min. per sq. meter at 37° C. and the prevailing barometric pressure (5). The hemoglobin concentration was measured with a photoelectric colorimeter. Heparin was used as the anti-coagulant to determine the hematocrit reading.

RESULTS

Twenty-four sets of observations were made on 18 anemic subjects (Table I). When the hemoglobin concentration was below 7 grams per 100 ml. and the hematocrit reading was below 20, the cardiac output in the resting basal state was usually increased. In the 9 experiments in which the hematocrit reading was below 14 and the hemoglobin below 5 grams, the average cardiac index was 6.5. In the 5 patients with hemoglobin concentration between 5 and 7 grams per 100 ml., the cardiac index averaged 4.7; in the 5 between 7 and 9 grams, it averaged 4; and in the 5 patients between 9 and 13 grams, it averaged 3.1.

At the bottom of Table I are given the average values for the various aspects of the circulation in patients with severe anemia (hemoglobin below 7 grams), in patients with moderate anemia (hemoglobin over 7 grams), and in a group of normal young male subjects. The cardiac output, the arteriovenous oxygen difference, and the atrial pressure readings were not significantly altered by moderate chronic anemia. The values for oxygen consumption, metabolic rate, ventilation, femoral arterial pressure, and pulse rate tended to be slightly higher in the moderately anemic than in the normal control group. This is probably not the result of moderate anemia, but of different criteria used in selecting the persons composing the two groups. The moderately anemic subjects

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Emory University School of Medicine.

were a heterogeneous group of patients of varying ages in whom minor degrees of vascular disease may have been present and in whom it was impossible to eliminate a certain amount of apprehension connected with the experiment. For these reasons, the moderately anemic group is a better control series to determine the effects of anemia on certain aspects of the circulation than is the group of carefully selected normal males. In the severely anemic patients, the diastolic and mean arterial pressures and the peripheral resistance were lower than in the moderately anemic subjects. There was no significant difference in the atrial pressure readings in the two groups.

To be certain that the differences observed between the patients with moderate and severe anemia were actually caused by anemia and were not the result of anxiety or of chance selection of patients who normally had a hyperactive circulation, observations were made on 4 very anemic subjects before and after the hemoglobin level had been doubled by transfusions or by the administration of liver extract. The pulse rate always fell, the systolic and diastolic arterial pressures rose, the atrial pressure either remained unchanged or increased, and there was a dramatic increase in peripheral resistance. The arteriovenous oxygen difference always increased and the cardiac output decreased. These studies confirm the trends observed in comparing the circulation of normal subjects with the circulation of anemic patients.

DISCUSSION

When the hemoglobin concentration falls below 7 grams per 100 ml., requirements of the body for blood are increased (Figure 1). At low levels of hemoglobin, one observes that circulatory insufficiency develops while the cardiac output is still markedly above that found in normal subjects under similar conditions. For this reason, the finding of a normal cardiac output does not necessarily mean that a given patient is not in shock. The output must be evaluated in terms of the requirements of the body for blood. An analogous situation is found in certain patients with congestive heart failure and anemia. Congestive failure may occur while the heart is pumping more than the usual amount of blood. A

cardiac output which is sufficient for a normal resting subject is insufficient for the anemic subject at rest and signs of congestive failure develop.

The heart rate tended to be elevated in anemia, but there was no obvious relationship between the pulse rate and the cardiac output in the series as a whole. In the 4 patients who were studied before and after increasing the hemoglobin level, both a fall in heart rate and cardiac output occurred.

There was no correlation between the atrial pressure and the cardiac output in these patients. In spite of the marked increase in cardiac output, there was no significant rise in atrial pressure. In 2 of the subjects who were studied before and after therapy for the anemia, the atrial pressure tended to rise as the volume of red cells increased and the cardiac output decreased. These data are in accord with our observations on moderate blood loss in which it was demonstrated that, within a certain range, the atrial pressure could be varied without changing the cardiac output (6). It was also found that anxiety caused a marked increase in cardiac output without an accompanying rise in atrial pressure (7).

The data reported here are in accord with other observations recorded in the literature. In a study of the effects of bleeding on the cardiac output in dogs, two workers (8) noted that individual dogs reacted to comparable degrees of anemia with considerable difference in the degree of increase in cardiac output. They state that in many animals a hemoglobin level of 60 to 70 per cent did not cause a rise in output, but that in others, with much milder anemia, the cardiac output was increased. They suggest that the more rapidly a given degree of anemia is produced the more is the output of the heart increased.

There has been no uniform agreement as to the level to which the hemoglobin concentration falls before the cardiac output is definitely increased. One author (9) noted no increase in cardiac output until the hemoglobin fell to around 50 per cent. Another (10) found a hyperactive circulation when the hemoglobin was below 50 per cent. Still others (11) believed that a considerably less degree of anemia caused a marked rise in cardiac output. In our patients, there was consistent increase in cardiac output when the

TABLE I
Observations on the circulation in 18 patients with chronic anemia

Patient	Diagnosis	Age years	Sex	Weight kgm.	Surface area sq. m.	Comment	Ventilation		Oxygen consumption ml. per min. per sq. m.	Metabolic rate— normal basal per cent	Oxygen content			Cardiac output l. per min.	Cardiac index l. per min. sq. m.	Hemoglobin grams per 100 ml.	Hematocrit reading	Blood pressure			Pulse rate beats per min.	Right atrial pressure mm. H ₂ O	Peripheral resistance abso- lute units	
							l. per min. sq. m.	l. per min. per sq. m.			Arterial	Mixed Venous	A-V diff.					Systolic	Diastolic	Mean				
J.F.	Aplastic anemia	43	M	65.8	1.84	Control After 750 ml. R.B.C. 3 months later	6.2 5.0 4.6	148 127 130	+13 -3 -1	3.6 7.5 4.6	1.6 4.6 2.9	2.0 2.9 2.4	13.6 7.9 4.3	7.4 4.3 5.4	2.4 5.4 4.1	5.6 14.5 11.4	56 77 79	116 131 131	62 70 70	77 88 88	115 94 65-70	70 65-70	450 863 640	
W.B.	Pernicious anemia	67	M	60.3	1.78	Control After 3 weeks liver therapy	4.6 5.1	143 190	+19 +55	4.5 10.3	2.0 6.8	2.5 3.5	9.5 6.6	5.7 4.0	3.5 8.2	5.7 4.0	8.6 10.3	86 29.8	113 153	46 80	71 106	81 65	35 35	600 1260
I.B. R.T.	Pernicious anemia Anemia secondary to blood loss	32	F	46.0	1.43	Control Day after 2000 ml. whole blood	3.9 4.0	142 143	+14 +13	4.4 5.3	2.1 3.5	2.3 1.8	11.9 13.0	8.3 7.9	3.3 4.3	10.3 13.4	10.3 94	108 94	49 53	71 73	101 115	65 45	470 450	
F.K. E.W.	Secondary anemia Sickle cell anemia	21 17	M F	65.8 56.7	1.87 1.66	2 weeks later Control Following day after 1000 ml. R.B.C.	3.5 4.0 4.7	125 148 157	-10 +14 +23	6.5 5.8 4.1	3.1 3.4 2.0	3.4 2.4 2.1	6.9 10.3 12.1	3.7 6.2 7.5	3.5 4.5 5.1	10.6 14.2 7.8	26.4 10.6	106 138	59 68	71 95	100 75	105 35	850 740	
R.C.	Aplastic anemia	50	M	60.3	1.62	Control Following day after 1000 ml. R.B.C.	4.0 3.2 4.2	135 175 159	+6 +45 +20	9.2 6.3 6.8	4.6 3.5 4.7	4.6 2.8 2.1	4.7 8.6 13.1	2.9 6.3 7.6	6.9 4.5 6.4	20.2 10.6 21.0	20.2 10.6 21.0	154 120 134	71 48 60	97 67 84	55 91 79	105 30 15	1690 620 510	
M.A. A.H.	Pernicious anemia Secondary anemia	41 30	F F	53.1 60.3	1.37 1.56	Control Day after 2000 ml. whole blood	4.0 3.2 4.2	135 175 159	+6 +45 +20	9.2 6.3 6.8	4.6 3.5 4.7	4.6 2.8 2.1	4.7 8.6 13.1	2.9 6.3 7.6	6.9 4.5 6.4	20.2 10.6 21.0	20.2 10.6 21.0	154 120 134	71 48 60	97 67 84	55 91 79	105 30 15	1690 620 510	
J.E.B. J.M.	Sickle cell anemia Pernicious anemia	39 37	F M	59.0 49.1	1.72 1.48	Control Day after 2000 ml. whole blood	4.2 3.8	159 153	+20 +27	6.8 4.7	4.7 5.4	2.1 3.7	13.1 6.3	7.6 3.5	6.4 7.5	21.0 27.2	21.0 27.2	134 156	60 86	84 111	79 88	15 20	1143 510	
L.W. L.B.	Secondary anemia Secondary anemia	19 45	F F	71.7 41.7	1.80 1.59	Control Day after 2000 ml. whole blood	4.2 3.4	131 105	-17 -9	9.1 11.9	3.7 9.0	3.7 2.9	6.3 5.7	3.5 3.6	7.5 8.8	27.2 32.7	27.2 32.7	156 120	86 62	111 90	88 74	20 25	1110 990	
R.H. R.H.	Secondary anemia Secondary anemia	29 19	F F	59.0 41.0	1.56 1.30	Control Day after 2000 ml. whole blood	3.5 3.9	108 143	+15 +15	13.1 13.7	9.3 8.9	3.8 4.7	5.7 3.0	3.6 3.0	9.9 10.7	36.5 36.5	36.5 36.5	131 100	63 65	70 75	75 100	35 35	1550 1550	
O.F. D.H.	Secondary anemia Secondary anemia	41 24	F M	81.2 52.2	2.0 1.55	Control Day after 2000 ml. whole blood	4.3 6.3	132 128	-9 +6	12.5 13.2	8.3 8.1	4.2 5.1	3.9 3.9	3.2 2.5	11.2 10.8	32.7 32.7	32.7 32.7	155 149	99 78	119 78	76 63	35 35	1550 1550	
Averages on patients with hemoglobin below 7 grams							4.2	144	+13	14.9	11.1	3.7	6.2	3.9	13.0	32.7	32.7	131	70	91	60	30	1176	
Averages on patients with hemoglobin 7.0 to 13.0 grams per 100 ml.							4.2	144	+13	2.6	3.8	3.3	5.7	3.6	9.7	32.7	32.7	121	57	79	83	43	716	
Averages on normal subjects							4.2	133	+5	3.8	3.8	3.3	3.6	3.6	9.7	32.7	32.7	131	72	90	79	36	1303	
							3.4	128	-7	4.0	4.0	3.3	3.3	3.3	9.7	32.7	32.7	124	67	85	68	31	1160	

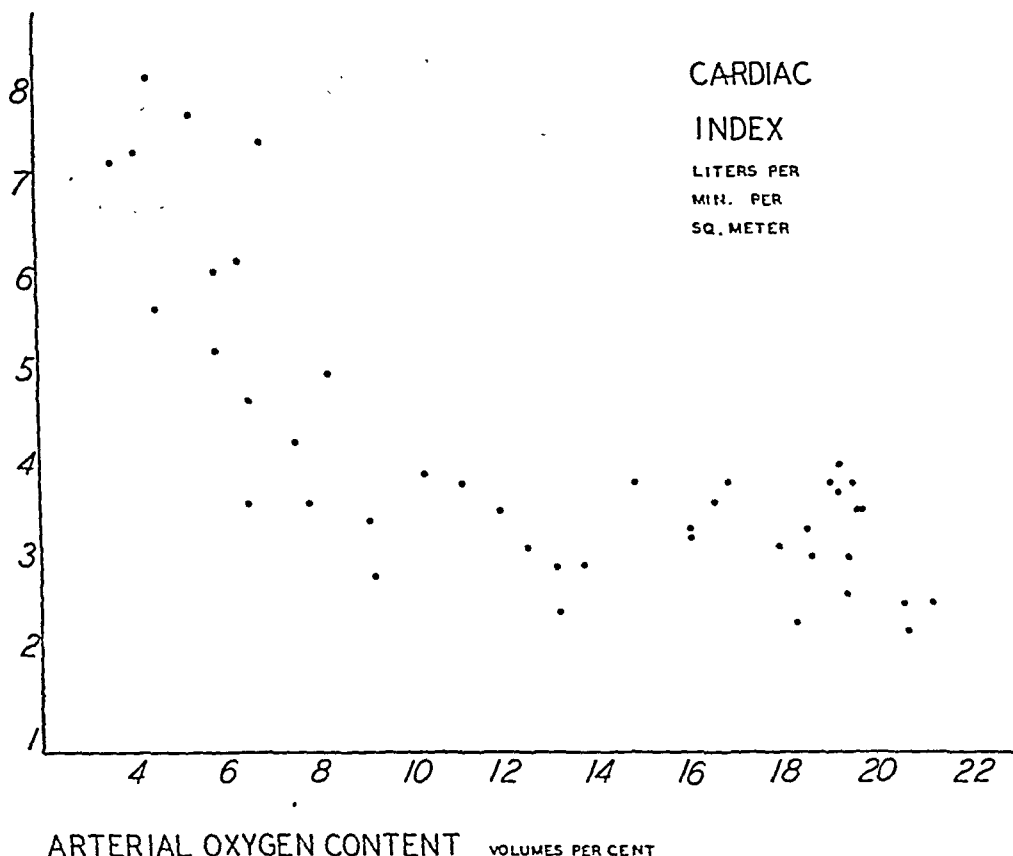


FIG. 1. RELATIONSHIP BETWEEN THE CARDIAC INDEX AND THE OXYGEN CONTENT OF THE ARTERIAL BLOOD

hemoglobin concentration was less than 7 grams per cent. This corresponds to a hemoglobin of around 50 per cent.

SUMMARY

1. Twenty-four sets of data were obtained on 18 anemic subjects. Atrial pressure readings and samples of mixed venous blood for determination of the cardiac output were obtained through a catheter introduced into the right atrium. The femoral arterial pressure was recorded optically by the method of Hamilton.

2. No consistent change in the circulation was observed when the hemoglobin level was above 7 grams per 100 ml. Below that level, the cardiac output at rest was increased, the arteriovenous oxygen difference and the peripheral resistance were decreased. The atrial pressure was not changed.

3. In 4 patients, observations were made before and after the hemoglobin had been doubled by transfusion or by the administration of liver extract. In each patient, the pulse rate fell, the diastolic and mean arterial pressures and peripheral resistance rose, and the cardiac output decreased.

4. When the hemoglobin level falls below 7 grams per 100 ml., the requirements of the body for blood are increased. This explains why anemic subjects may have circulatory insufficiency manifested by either shock or heart failure, while the heart is pumping as much or more blood as would be required by a normal subject under similar conditions.

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THE EFFECT OF VENESECTION AND THE POOLING OF BLOOD IN THE EXTREMITIES ON THE ATRIAL PRESSURE AND CARDIAC OUTPUT IN NORMAL SUBJECTS WITH OBSERVATIONS ON ACUTE CIRCULATORY COLLAPSE IN THREE INSTANCES¹

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The mechanisms by which the body compensates for a decrease in blood volume have not been thoroughly studied in man. All physicians are aware that from 500 to 1000 ml. of blood can be removed from a person of average size without producing any symptoms, if the body is horizontal. If the blood is removed rapidly, hemodilution plays little part in the immediate adjustment. Do the arterioles constrict, maintaining the arterial pressure at a normal level at the expense of blood flow to the tissues? Is the atrial pressure maintained at the normal level by venous constriction? Or is it possible that up to a certain point, the heart can function effectively with a falling atrial pressure so that the circulation can be maintained without either arteriolar or venous constriction in spite of a moderate decrease in blood volume?

The purpose of this paper is to describe the changes in the circulation in normal subjects caused by moderate loss of blood from the body, either as the result of venesection or by pooling blood in the extremities through the application of venous tourniquets. During the experiments, acute circulatory collapse (primary shock) occurred in three instances and quantitative observations were made on the changes occurring in the circulation.

METHODS

Medical students, physicians, and paid volunteers served as subjects. They had not eaten for the preceding 12 hours and they rested in the horizontal position for at least 30 minutes before the experiment began.

A catheter was introduced through the antecubital vein into the right atrium for obtaining samples of mixed venous blood and for recording the mean atrial pressure in mm. H₂O (1, 2). In the subjects who were to be bled, a needle was placed in the femoral artery for recording the arterial pressure (3) and for obtaining samples of arterial blood. The mean arterial pressure was measured by planimetric integration of the area beneath the tracing. When tourniquets were applied to the upper thighs, arterial blood was not obtained. In these cases, a sample of atrial blood was saturated with oxygen and its oxygen content determined. It was assumed that the arterial blood was 95 per cent saturated. At the time each cardiac output was measured, the figure for the oxygen content of the arterial blood was changed in proportion to the change in the hemoglobin concentration or hematocrit reading of the sample of atrial blood used in calculating the cardiac output at that particular time. Two-minute samples of expired air were collected in Douglas bags and their oxygen and carbon dioxide contents were determined by the method of Haldane.

The oxygen content of the blood was measured by the method of Van Slyke and Neill (4). Heparin was the anticoagulant used for hematocrit determinations. The peripheral resistance was recorded in absolute units by the formula:

$$R = \frac{P_m \text{ (mean pressure in mm. Hg)} \times 1332}{C.O. \text{ (cardiac output in ml. per sec.)}} \quad (5)$$

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Emory University School of Medicine.

TABLE I

Observations on the effect of removal of 300 to 900 ml. of blood from normal subjects

Subject	Weight	Surface area		Ventilation	Oxygen consumption	Arterial O ₂ content	Mixed venous O ₂ content	A-V oxygen difference	Cardiac output	Cardiac index	Arterial pressure			Pulse rate	Atrial pressure	Peripheral resistance
											Systolic	Diastolic	Mean			
	kgm.	sq. m.		l. per min. per sq. m.	ml. per min. per sq. m.	volumes per cent			l. per min.	l. per min. per sq. m.	mm. Hg			beats per min.	mm. H ₂ O	absolute units
ES	82.6	2.01	Control	3.1	109	18.3	13.7	4.6	4.9	2.4	136/73	91	61	55	1500	
			Removal 700 ml. blood	3.5	125	18.3	14.8	3.5	7.4	3.6	125/66	90	71	0	972	
WM	72.6	1.90	Control	2.4	103	19.4	16.2	3.2	6.1	3.2	116/62	81	60	45	1062	
			Removal 300 ml. blood	2.5	105	19.4	16.1	3.3	6.1	3.2	122/70	86	60	25	1126	
WLM	82.1	2.04	Control	2.7	121	21.2	16.5	4.7	5.3	2.6	112/55	71	60	35	1085	
			Removal 500 ml. blood	2.9	121	20.9	15.9	5.0	4.9	2.4				0		
CG	68.9	1.91	Control	3.5	131	18.5	14.7	3.8	6.6	3.4	141/75	96	72	35	1160	
			Removal 500 ml. blood								133/72	90	76	5		
			Collapse 30 minutes later	11.1	159	18.0	12.9	5.1	6.0	3.1	61/27	37	48	25	493	
WP	68.0	1.74	Control—Feb. 16, 1944	4.9	142	16.0	11.9	4.1	6.0	3.4	143/75	98	68	50	1305	
			Removal 900 ml. blood	4.0	143	15.7	11.4	4.3	5.7	3.3	136/80	100	80	0	1400	
			Collapse 500 ml. gelatin solution	5.7	139	15.5	11.3	4.2	5.8	3.3	83/38	56	56	20	772	
WP	68.0	1.74	Control—Oct. 25, 1943	4.9	152	15.5	11.8	4.7	5.6	3.2	91/51	64	68	5	917	
			Removal 500 ml. blood	4.8	162	15.6	12.4	3.2	8.8	5.1	127/72	95	75	40	863	
			Blood returned	5.2	190	15.6	11.7	3.9	8.5	4.9	102/42	61	56		573	
WP	68.0	1.74	Control—Dec. 2, 1943	4.1	172	15.4	11.6	3.8	7.9	4.5	120/68	86	75		870	
			Removal 500 ml. blood	3.3	153	14.5	11.8	2.7	9.9	5.7	141/80	100	80	45	812	
			Blood returned	3.4	152	14.4	11.7	2.7	9.8	5.6	135/80	101	84	0	824	
JT	70.8	1.84	Control	4.1	157	14.4	11.7	2.7	10.1	5.7	138/72	97	75	45	762	
			Removal 500 ml. blood	4.6	181	18.5	15.6	2.9	11.5	6.2	165/87	115	107	40	800	
			Blood returned	4.9	173	17.1	14.2	2.9	11.1	6.0	140/81	101	100	-10	727	
WB	75.8	1.93	Control	4.8	154	17.5	15.1	2.4	12.2	6.6	137/79	99	100	50	656	
			Removal 500 ml. blood	2.6	146	20.2	18.3	1.9	14.8	7.7	128/66	88	100	0	475	
				3.0	144	20.2	16.8	3.4	8.1	4.2	128/71	88	90	-30	868	
IR	72.6	1.84	Control	4.6	160	18.4	15.5	2.9	10.2	5.5	167/94	123	88	30	786	
			Removal 800 ml. blood	4.7	177	17.8	13.7	4.1	7.9	4.3				5		
			After 500 ml. gelatin solution	4.6	157	15.8	12.5	3.3	8.8	4.8				40		

relaxed. The average decrease in atrial pressure was 38 mm. H₂O. In subject ES, after removal of 700 ml. of blood, the pulse rate increased 7 beats per minute and the oxygen consumption rose. The arteriovenous oxygen difference became less and the cardiac output was considerably increased in spite of a decrease in atrial pressure of 55 mm. H₂O. The mean femoral arterial pressure did not change and the peripheral resistance was considerably decreased. It was felt that the rise in cardiac output resulted from apprehension induced by the removal of blood. In subject WM, only 300 ml. of blood were removed. The atrial pressure fell 20 mm. H₂O, but there was no significant change in the arteriovenous oxygen difference, oxygen consumption, cardiac output, mean arterial pressure, or peripheral resistance. In subject WLM, the atrial pressure fell 35 mm. H₂O after the removal of 500 ml. of blood.

The arteriovenous oxygen difference increased 0.3 volume per cent and the cardiac output fell 0.4 liter per minute. These changes are not significant.

In subject CG, the atrial pressure fell 30 mm. H₂O after the removal of 500 ml. of blood. Immediately thereafter the heart rate had risen from 72 to 76 and the mean arterial pressure had fallen from 96 to 90 mm. At this time, blood pressure cuffs which had been previously applied to the upper thighs were inflated to 80 mm. Hg. In a short time, the subject became pale and began to sweat. The tourniquets were immediately released, but the pallor became more marked and nausea began. The subject remained conscious, but he hyperventilated until his hands and feet felt numb. The heart rate had fallen to 48 beats per minute and the mean arterial pressure had decreased from 90 to 37 mm. Hg. The cardiac

output had fallen from 6.6 to 6.0. This is not a significant change. The peripheral resistance was strikingly lowered. Because of the hyperventilation, the atrial pressure reading was not entirely satisfactory. A reading of 25 mm. H_2O was recorded. This would mean a rise rather than a fall in atrial pressure during the circulatory collapse. Thirty minutes later the subject had recovered. The atrial pressure was 10 mm. lower than the control reading and the mean arterial pressure remained appreciably depressed. The oxygen consumption had decreased, and the arteriovenous oxygen difference and the cardiac output were essentially the same as before the venesection.

Subject WP was being bled for the third time. The atrial pressure was lowered 50 mm. H_2O by a venesection of 900 ml. Observations made immediately after the removal of the blood showed no change in the circulation except a rise in pulse rate. Nine minutes later the subject became ashen, began to sweat, and complained of nausea. The atrial pressure rose above the level recorded immediately after bleeding. The heart rate slowed, the mean pressure fell from 100 to 56 mm. Hg, and the peripheral resistance decreased 50 per cent. The oxygen consumption, the arteriovenous oxygen difference and the cardiac output remained unchanged. Five hundred ml. of gelatin solution were given intravenously. The patient developed urticaria and hemodilution did not occur. The atrial pressure remained low, the mean arterial pressure and the peripheral resistance were depressed, the metabolism rose somewhat, and the cardiac output remained unchanged.

Effect of venesection in subjects with hyperactive circulation. Five venesections were performed on 4 subjects who had hyperactive circulations before the venesections. Five hundred ml. (800 ml. from IR) of blood were removed each time and the average fall in atrial pressure was 38 mm. H_2O .

The first 2 experiments were carried out on subject WP. On October 25, 1943, the venesection was followed by acute circulatory collapse. This was not as severe as occurred in the same subject on February 16, 1944. The atrial pressure was not measured after the venesection. The metabolism rose, the arteriovenous oxygen differ-

ence increased, and the cardiac output remained unchanged. As in the other 2 instances of acute circulatory collapse, the heart rate slowed, the systolic and diastolic pressures and the peripheral resistance fell. The blood was returned to the subject and measurements of the circulation showed the mean arterial pressure still to be somewhat depressed and the cardiac output to have fallen slightly. The change in cardiac output, which is probably not significant, may have been the result of progressive relaxation of the subject, rather than of a change in blood volume. On December 2, 1943, the experiment was repeated. The control cardiac output was greatly increased above the expected resting level. Venesection produced a fall in atrial pressure of 45 mm. H_2O with an increase in heart rate of only 4 beats per minute. There was no change in cardiac output, mean arterial pressure, or peripheral resistance. The blood was returned without any significant change, except that the atrial pressure returned to the control level.

In subject JT, the removal of 500 ml. of blood caused a fall in atrial pressure of 50 mm. H_2O without any change in cardiac output. The heart rate decreased 7 beats per minute and the mean arterial pressure fell 14 mm. Hg. These changes were attributed to lessened apprehension, rather than to changes in blood volume, because they persisted after the blood was returned to the subject and the atrial pressure had risen to the level prior to bleeding. There was a progressive fall in oxygen consumption which was attributed to lessening of anxiety.

In subject WB, the atrial pressure fell 30 mm. H_2O . The heart rate decreased 10 beats per minute. The cardiac output fell from 14.8 to 8.1 liters per minute and the peripheral resistance doubled. It was felt that the high cardiac output, the fast pulse rate, and the low peripheral resistance were other manifestations of the nervous tension which the subject exhibited during the experiment. The question rose as to whether the cardiac output decreased because the atrial pressure decreased, or because the subject became more relaxed. Several months later the cardiac output was again measured under basal conditions. The heart rate was 82 beats per minute, the cardiac output was 7.6 liters per minute, and the peripheral resistance was 540. There being no

suggest that the change in cardiac output noted in the first experiment was caused by relaxation, rather than by the venesection.

In the eighth subject (IR), the atrial pressure fell 25 mm. H₂O and the cardiac output fell from 10.2 to 7.9 liters per minute. The cardiac output, after the venesection, was slightly above the expected resting value and again the question rose as to whether the fall resulted from the venesection or from relaxation because the venesection was completed. Five hundred ml. of gelatin solution were given intravenously and the atrial pressure returned to the control level. The cardiac output was between the value obtained before and that obtained immediately after venesection.

Effects of venous tourniquets. In 4 subjects, studies on the circulation were made before and after the inflation of venous tourniquets applied to the proximal portions of the thighs. The pressure in the tourniquets ranged from 70 to 80 mm. Hg. In subject CB, the atrial pressure fell 50 mm. H₂O. At the end of 10 minutes, the arteriovenous oxygen difference had increased and the cardiac output had decreased. The tourniquets were released and the atrial pressure returned to the control level. Fifteen minutes later, the arteriovenous oxygen difference and the cardiac output were at the level which they had

reached while the tourniquets were inflated. In subject PM, the atrial pressure fell 35 mm. The arteriovenous oxygen difference decreased and the cardiac output may have increased slightly. After the release of tourniquets, the changes in the arteriovenous oxygen difference and cardiac output persisted. In subject JC, the atrial pressure fell 40 mm. The arteriovenous oxygen difference increased and the cardiac output tended to fall. In subject SL the atrial pressure fell 65 mm. The metabolism increased so that the cardiac output was increased, though the arteriovenous oxygen difference remained unchanged.

The pooling of blood in the extremities produced a fall in atrial pressure equal to that produced by the removal of 500 to 1000 ml. of blood by venesection. This was to be expected from earlier data (6) which showed that between 500 and 900 ml. of blood may be pooled in 2 lower and 1 upper extremities by the use of venous tourniquets. As in the bleeding experiments, there was no demonstrable correlation between the atrial pressure and cardiac output. The data on subjects CB and PM demonstrate the fact that erroneous conclusions may be drawn unless determinations are made before, during, and after the application of the tourniquets to exclude psychic effects.

TABLE II

The effect on the circulation of pooling blood in the extremities by venous tourniquets

Subject	Weight	Surface area		Ventilation	Oxygen consumption	Arterial			Cardiac output	Cardiac index	Arterial pressure		Pulse rate	Atrial pressure
						O ₂ content	Mixed venous O ₂ content	A-V oxygen difference			Systolic	Diastolic		
	kgm.	sq. m.		l. per min. per sq. m.	ml. per min. per sq. m.	volumes per cent			l. per min.	l. per min. per sq. m.	mm. Hg	beats per min.	mm. H ₂ O	
CB	68.9	1.90	Control	2.3	123	18.6	15.6	3.0	7.8	4.1	120/70	68	40	
			Tourniquets on 10 min. at 70 mm. Hg	2.3	118	19.2	15.1	4.1	5.5	2.9	110/70	72	-10	
			15 minutes after release	2.5	127	19.1	14.9	4.2	5.7	3.0	112/70	64	35	
PM	69.6	1.78	Control	3.8	143	19.6	15.6	4.0	6.4	3.6	120/64	73	25	
			Tourniquets on 6 min. at 80 mm. Hg	3.7	141	19.1	15.5	3.6	6.9	3.9			-10	
			5 minutes after release			19.3	15.7	3.6	6.9	3.9			25	
JC	56.2	1.69	Control	4.0	130	18.5	13.8	4.7	4.7	2.8	120/63	58	25	
			Tourniquets on 7 min. at 80 mm. Hg	4.3	129	17.9	12.6	5.3	4.1	2.4			-15	
SL	66.2	1.76	Control	3.0	114	16.8	13.5	3.3	6.2	3.5		64	80	
			Tourniquets on 6 min. at 80 mm. Hg	3.4	130	16.8	13.4	3.4	6.7	3.8		64.		

DISCUSSION

The removal of blood in 300 to 900 ml. quantities, by venesection or the pooling of blood in the extremities by venous tourniquets, caused a consistent fall in the atrial pressure. There was no close correlation between the removal or pooling of blood with any other function of the circulation which was measured. When the resting heart rate was slow, it usually rose slightly. The mean arterial pressure, the peripheral resistance, the arteriovenous oxygen difference, and the cardiac output showed no consistent changes. As pointed out previously, the method used in these studies is not sufficiently accurate to detect small changes in cardiac output; but no difficulty was encountered in demonstrating the 20 per cent decrease in cardiac output produced by tilting subjects to the erect position (10).

The question arose as to whether the effective atrial pressure actually fell as the blood was removed in moderate amounts. Could it be that the intrathoracic pressure showed a simultaneous fall as blood was removed, so that in spite of the apparent fall in atrial pressure the effective filling pressure remained unchanged? It has been shown that venous tourniquets decrease the amount of blood in the thorax (7). The intrathoracic pressure was therefore measured directly in subject ES, who had previously been shown to have a decrease in atrial pressure without a fall in cardiac output. The application of venous tourniquets caused a fall in atrial pressure of 70 mm. H_2O ; the intrathoracic pressure varied between 0 and -1 cm. of H_2O before and 0 and -2 cm. H_2O after the inflation of the tourniquets. It was concluded, therefore, that the fall in atrial pressure was not compensated for by a considerable increase in negative intrathoracic pressure.

The atrial pressure was recorded optically by the method of Hamilton, before and after the venesection. The lowered mean pressure was not accompanied by a rise in the atrial systolic pressure.

From these data, it appears that the normal atrial pressure is somewhat in excess of that required to fill the ventricle at rest, and that, therefore, a moderate fall in atrial pressure can occur without any disturbance in cardiac output.

The fact that there was no evidence of generalized arteriolar vasoconstriction after the removal of a moderate amount of blood came as no surprise. Plethysmographic studies had failed to show any change in blood flow of the forearm as blood was pooled in the lower extremities by venous tourniquets (8). Furthermore, the fact that the ordinary donor suffers no inconvenience after the removal of 500 ml. of blood, and shows no clinical signs of poor tissue blood flow during the time his blood volume is depleted, is strong argument against a significant degree of arteriolar constriction with resultant ischemia of the tissues. Because of these observations, we taught our students that constriction of the veins and venules was the probable compensatory mechanism which maintained the atrial pressure and cardiac output at a constant level as the blood volume was decreased. To our surprise, it appears that no compensatory mechanism exists to maintain a constant atrial pressure within the observed limits. The heart seemed able to pump blood normally with a falling atrial pressure. This was true even when the cardiac output had been greatly elevated by anxiety. It is to be emphasized that these observations were all made on subjects from whom relatively small amounts of blood had been removed. In massive hemorrhage, various compensatory mechanisms and a fall in cardiac output can be demonstrated.

This lack of correlation between cardiac output and atrial pressure was noted in comparing the cardiac output of patients with severe anemia with that of normal subjects. A 100 per cent increase in cardiac output occurred without a significant change in atrial pressure (9). Likewise, in a study of normal subjects, it was noted that anxiety caused a striking rise in cardiac output without a rise in atrial pressure (10).

In a study of the effects of hemorrhage on normal subjects, certain investigators (11) found only slight changes in blood pressure, pulse rate, and ballistocardiograph tracing after the removal of 500 to 1000 ml. of blood. While they did not measure the atrial pressure, we can be certain from our observations that sufficient blood was removed to cause a significant lowering of pressure.

Other workers (12) reported that the removal

of blood caused a parallel fall in cardiac output and atrial pressure. These authors used the catheter technique for obtaining blood samples and pressure readings from the right atrium. They also found that lowering of the atrial pressure by application of venous tourniquets to the thighs caused a parallel fall in atrial pressure and cardiac output. These same authors noted that epinephrine caused an increase in cardiac output in the presence of a falling venous pressure.

The acute circulatory collapse which developed on 3 occasions during our observations was characterized by a marked fall in systolic, diastolic, and mean arterial pressure, without a demonstrable change in cardiac output. The venous side of the circulation did not seem to be affected. In the one experiment in which satisfactory measurements were made repeatedly, the atrial pressure, with the onset of the circulatory collapse, increased above the level to which it had fallen immediately after the venesection. In 2 experiments, the arteriovenous oxygen difference increased, but as the oxygen consumption also increased, the cardiac output remained unchanged. It might be argued that the rise in metabolism without a corresponding rise in cardiac output produced a state of relative circulatory insufficiency, despite the fact that there was no actual fall in the cardiac output. In the third instance, however, the metabolism did not rise.

One worker (13) has made observations on the atrial pressure and cardiac output in one subject who developed acute circulatory collapse after the removal of 900 ml. of blood. "The blood pressure fell from 110 to 58 and the pulse from 82 to 50. During the faint the right atrial pressure rose by 2 cm. from the low level reached at the end of the hemorrhage. The cardiac output also rose slightly (3.2 to 3.6 liters per minute)." He concluded that this type of circulatory failure is due to a sudden loss of peripheral resistance (presumably arteriolar tone). Further observations on 7 subjects have supported this conclusion (14).

It is probable that the venesection had nothing to do with the circulatory collapse in the subjects reported here, except inasmuch as it served as a psychic and reflex stimulus. The data demonstrated that the venesection of this size does not cause a fall in cardiac output. Observations in

many laboratories and blood donor centers have shown that this type of circulatory collapse frequently occurs before any blood is withdrawn.

Because of the marked fall in arterial pressure and the striking pallor, we were surprised to find that the cardiac output had not fallen. The fact that the cardiac output with the subject in the horizontal position remained unchanged with acute circulatory collapse may be the reason that this type of circulatory disturbance is so benign. On standing, these subjects are very apt to lose consciousness. Whether this results from a more striking arteriolar dilatation with too low a pressure to raise blood to the head, or from venous pooling with a subsequent fall in atrial pressure and a further decreased arterial pressure, because of a drop in cardiac output, has not been determined.

The question arises as to whether the fall in arterial pressure causes the pallor, epigastric distress, sweating, weakness, and nausea, or whether both the decrease in arterial pressure and the other symptoms are the result of intense stimulation of the autonomic nervous system from reflexes arising either in a part of the body or from emotional content of thought. Clinical observations have shown that the pallor, epigastric distress, sweating, weakness, and slow pulse rate may, or may not, be accompanied by a marked fall in arterial pressure. It has also been noted that these symptoms may persist long after the arterial pressure has returned to normal or has been raised by the administration of vasoconstrictor drugs. The sudden circulatory failure produced by heart block from organic disease, or by cardiac standstill from carotid sinus pressure, is not associated with nausea or sweating, though moderate pallor may occur. Patients with true orthostatic hypotension have a striking fall in arterial pressure when they stand, which may be sufficiently severe to cause unconsciousness. This fall in arterial pressure is a true postural effect and is not appreciably influenced by emotional stimuli. These patients do not show pallor, sweating, or nausea as the arterial pressure falls (15). After an anaphylactic reaction or after a bout of fever, the arterial pressure may be markedly lowered without the other symptoms which commonly occur with acute circulatory collapse. All of these observations support the assumption that the entire syndrome

of pallor, nausea, weakness, sweating, slow pulse rate, and fall in arterial pressure results from reflex stimulation of the autonomic nervous system and that the fall in arterial pressure accompanies, rather than causes, the other symptoms.

The above hypothesis does not explain certain other observations. Standing upright makes subjects much more likely to have acute circulatory collapse. Indeed, after the collapse has occurred and the patient has recovered in the horizontal position, it is frequently possible to reproduce the syndrome by motionless standing. Furthermore, in patients who have bled profusely, syncope can be easily induced by tilting the subject with the feet down. It is usually preceded by pallor, sweating, nausea, and a sharp fall in arterial pressure. In the past, we have wondered whether the symptoms, as well as the syncope, were produced by cerebral ischemia, resulting from a diminished cardiac output because of a decreased venous return secondary to venous pooling from gravity. It may be that the situation is much more complex and that under certain conditions standing causes an increase in activity of the autonomic nervous system, irrespective of a fall in cardiac output. If this produced a marked decrease in peripheral resistance and the arterial pressure became very low, syncope would occur from cerebral ischemia. This problem needs further study.

The realization that the circulatory collapse with the subject in the horizontal position is primarily a sudden reflex loss of peripheral resistance, and not circulatory failure secondary to a decrease in venous return, stimulates one to renew the search for effective drug therapy to prevent its occurrence in donors. Our impression is that a trial of therapy directed toward a lessening of generalized autonomic activity, rather than toward a production of arteriolar constriction, would be worthwhile.

SUMMARY AND CONCLUSIONS

1. The effect on the circulation of the removal of 300 to 900 ml. of blood by venesection and the effect of pooling of blood in the extremities by the venous tourniquets were studied in 12 normal males. The atrial pressure readings and the samples of mixed venous blood for measuring the

cardiac output were obtained through a catheter introduced into the right atrium by way of the antecubital vein. The femoral arterial pressure was recorded optically.

2. Venesection and the application of venous tourniquets caused a fall in atrial pressure from 20 to 65 mm. H₂O. There was no consistent change in cardiac output, mean arterial pressure, or peripheral resistance. Returning the blood by transfusion or by releasing the venous tourniquets was followed by a return of the atrial pressure to the control level without any change in the cardiac output.

3. The data suggest that the normal atrial pressure with the subject in the recumbent position is somewhat in excess of that required to fill the ventricles and that a decrease in blood volume of the degree reported here produces a lowering of atrial pressure without interfering with ventricular filling. These observations obviously do not apply to massive hemorrhage.

4. Acute circulatory collapse appeared in 3 instances. The subjects became pale, sweated profusely, and complained of nausea and weakness. The pulse rate slowed strikingly. The atrial pressure increased, the cardiac output remained unchanged. There was a marked fall in the arterial pressure and peripheral resistance. The circulatory collapse appeared to be the result of a sudden decrease in peripheral resistance because of reflex vasodilatation, presumably in the arterioles. There was no evidence of a decreased venous return.

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THE IMPORTANCE OF COMPENSATING VASOCONSTRICTION IN UNANESTHETIZED AREAS IN THE MAINTENANCE OF BLOOD PRESSURE DURING SPINAL ANESTHESIA¹

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The trauma of surgery or injury is regularly associated with alterations in the circulatory status of the individual. Such alterations may be limited to the site of injury, but if the injury is at all extensive, and especially if associated with hemorrhage and severe pain, a more generalized circulatory response may be expected. This response may involve change in cardiac output, volume of blood, constitution of blood, volume of extracellular fluid, circulation time, blood pressure, and peripheral circulation (1). When the response is inadequate, various types of circulatory insufficiency, such as peripheral vascular collapse or even shock, may supervene. Measurement of the response while it is proceeding is important in order that therapeutic measures may be employed as early as needed. Clinically, observation of the blood pressure is usually the sole quantitative criterion of the changing circulatory status, although attention is also paid to quality and rate of the pulse, the color and moisture of the skin, and depth and rate of respiration. The other constituents of the circulatory response to trauma are subject to measurement in human beings, but are usually neglected because they are not readily and conveniently ascertainable.

In the course of a study of the circulatory adjustments attendant upon surgical trauma, plethysmographic registration of the peripheral circulation has been utilized as an additional clinical guide to the circulatory response. This was undertaken in the belief that efforts to maintain blood volume, blood pressure, and cardiac output are directed toward the continuation of an adequate supply of blood to the tissues of the body. If the peripheral cutaneous circulation can be maintained, then, presumably, the circulatory adjustments are adequate to the needs of the more

important organs. It has been necessary to evaluate the effects of various types of anesthesia upon the peripheral circulation in view of the universal use of anesthesia in the course of major surgical operations. This report deals with the study of certain peripheral vascular adjustments during spinal and regional anesthesia.

APPARATUS AND METHODS

The pneumoplethysmograph (2) was used to record the rhythmic variations in the volume of the fingers and toes dependent upon constriction and dilatation of the small peripheral blood vessels (3). Such rhythmic pulsations are recorded as *pulse waves* synchronous with the cardiac beat and *alpha waves*. The latter occur irregularly at a rate 4 to 7 times per minute and vary in size up to 10 times that of pulse waves. The *pulse waves* may be as small as 1 to 2 c. mm. per 5 ml. of finger or toe or as large as 20 c. mm. Commonly, large alpha waves occur in association with varying, medium-sized, pulse waves (5 to 15 c. mm.) and small alpha waves, with very small or very large pulse waves (4).

Twenty-eight patients, ranging in age from 30 to 61 years, were subjected to spinal anesthesia for such surgical procedures as hemorrhoidectomy or herniorrhaphy. The observations reported here were made before the onset of surgical manipulation. The patients had no known hypertension or cardiac or peripheral vascular disease and were regarded as in good health except for their relatively minor surgical complaint. Twenty-four were male and 4 female. The spinal anesthetic agents employed were procaine hydrochloride (50 to 100 mgm.) or Monocaine Formate (75 to 150 mgm.). The intrathecal injection was made through the third or fourth lumbar intervertebral space while the patients were in the lateral position. For 2 of the patients, a malleable spinal puncture needle was inserted, and the patients then returned to a supine position, after which the anesthetic agent was injected. For the others, a regular spinal puncture needle was inserted, the anesthetic drug injected, the needle withdrawn, and the patient then returned to the supine position.

Plethysmograms were obtained from the index finger and second toe of the same side of the body. They were made with the patients supine and the fingers and toes were within 6 inches of the level of the heart. Plethysmograms were made before the insertion of the spinal puncture needle and after the injection of the anesthetic drug.

¹ This is the 14th paper reporting the results of studies of the small blood vessels and related subjects.

TABLE I

Comparison of the volume of pulse waves of the fingers and toes before and after spinal anesthesia unassociated with decrease in blood pressure

Number	Age	Sex	Blood pressure		Pulse rate		Pulse waves—toe		Pulse waves—finger		Upper limit of anesthesia
			Before anesthesia	After anesthesia	Before anesthesia	After anesthesia	Before anesthesia	After anesthesia	Before anesthesia	After anesthesia	
	<i>years</i>		<i>mm. Hg</i>	<i>mm. Hg</i>			<i>c. mm. per 5 ml.</i>		<i>c. mm. per 5 ml.</i>		
1	51	M	136/84	138/80	60	70	4.0	13.0	7.5	3.0	T-8
2	38	M	106/74	114/50	58	66	2.0	14.0	7.0	3.0	T-11
3	50	M	116/86	90/70	64	64	1.5	11.0	3.0	1.5	T-8
4	36	F	110/68	100/50	78	88	4.0	15.0	8.0	5.0	T-5
5	47	M	118/60	110/74	76	66	1.5	7.0	5.0	4.0	T-11
6	60	M	140/100	130/90	86	88	2.0	10.0	8.0	5.0	T-6
7	55	M	100/70	106/78	60	64	8.0	15.0	10.0	3.0	T-4
8	50	M	146/90	130/80	84	86	6.0	12.0	6.0	4.0	T-8
9	45	M	130/96	130/96	84	116	1.5	5.0	3.0	1.0	T-8
10	32	M	100/50	96/60	70	60	1.5	12.0	4.0	1.5	T-8
11	47	M	106/70	102/60	96	86	1.0	5.0	3.5	2.0	T-6
12	45	M	144/80	140/78	70	96	2.5	6.0	5.5	1.5	T-4
13	38	M	124/72	126/70	76	80	2.0	8.5	2.0	1.0	T-9
14	30	F	118/72	108/80	84	80	1.5	8.0	6.0	3.5	T-9
15	61	M	100/70	100/60	84	88	4.0	9.5	4.5	1.5	T-8
16	49	F	130/80	140/80	84	94	4.0	10.5	7.0	4.0	T-11
17	50	M	130/78	110/80	90	92	3.0	11.0	4.5	3.0	T-9
18	39	M	160/80	156/76	92	120	2.0	5.0	6.0	2.5	T-6
19	44	M	100/50	110/60	72	70	8.0	14.0	8.0	5.0	T-9
20	64	M	110/80	122/78	74	66	2.0	3.5	2.5	1.5	T-11
Average	47		121/76	118/73	77	82	3.1	9.8	5.6	2.8	

Measurements of blood pressure were made at 2- to 4-minute intervals by the use of an ordinary sphygmomanometer. Values for pulse rates were taken from the plethysmographic records. All of the studies were made in an environmental temperature within 5° of 75° F. Ephedrine sulfate (25 mgm.) was given intravenously to 8 of the patients in whom a progressive fall in blood pressure had occurred following the onset of spinal anesthesia.

RESULTS

The plethysmograms of fingers and toes obtained before the insertion of the spinal needle showed medium-sized pulse waves and large alpha waves in 19 of the subjects. Such records are suggestive of a state of moderate anxiety (4), not unexpected in patients about to be operated upon. In 9 patients, both the pulse waves and alpha waves were small, indicating generalized peripheral vasoconstriction associated with more intense anxiety. Plethysmograms recorded after injection of the drug and return of the patient to the supine position, but before the inception of anesthesia, showed the same pulse wave-alpha wave pattern as before the insertion of the spinal needle. Thus, at the time of onset of anesthesia, all of the subjects had plethysmographic tracings indicative of moderate

to relatively intense vasoconstriction in the fingers and toes.

During the course of spinal anesthesia, 20 patients exhibited no more than a slight change in systolic or diastolic blood pressure and in only 3 of these was there a change in pulse rate greater than 12 beats per minute. The upper level of sensory anesthesia in these patients ascended at least as high as the eleventh but not higher than the fourth thoracic dermatome. The plethysmographic records obtained from the fingers and toes of this group showed relatively consistent alterations in both the pulse and alpha waves. Within 2 to 5 minutes after injection of the drug, the pulse waves in the toe began to increase and within another few minutes attained great magnitude (Table I). The change in volume of the pulse waves was from an average value of 3.1 c. mm.² to an average value of 9.8 c. mm. Concurrently, the alpha waves were suppressed and the third simultaneous event was a marked increase in the total volume of the toe tip. In 1 subject, the volume change was as much as 200 c. mm. equalling $\frac{1}{25}$ of the total volume of the toe tip (Figure 1).

² All changes in volume are reported in c. mm. per 5 ml. of finger or toe tip.

COMPENSATING VASOCONSTRICTION IN SPINAL ANESTHESIA

occurring simultaneously with the changes in the toes, were alterations in the pulse e-alpha patterns in the finger tips. In none of these patients did the level of spinal anesthesia extend high enough to make it possible that the changes in the finger could be attributed to a direct effect of the anesthetic drug upon the sympathetic fibers passing from the spinal cord to the finger. The changes in the records of the fingers included: (1) a progressive decrease in the volume of the pulse waves from an average of 5.6 mm. to 2.8 c. mm.; (2) suppression of alpha waves; and (3) a gradual decrease in the total volume of the finger tip of a magnitude of about one third of the simultaneous increase in the total volume of the toe.

In 8 patients following the onset of anesthesia, a fall of systolic pressure of 40 to 80 mm. Hg occurred and was accompanied by a fall of about one half of this amount in the diastolic. In 4 of these patients (upper limit of sensory anesthesia T8, T4, T4, T4), the response of the toes was the same as that seen in the 20 subjects whose arterial tension remained unchanged; the response in the fingers differed in that instead of showing a decrease in size of pulse waves, there occurred an increase in pulse waves at the time of the fall

in blood pressure. When ephedrine sulfate administered intravenously, the pulse waves of the finger decreased in amplitude, thus re-establishing the pattern of vasoconstriction and restoring the blood pressure to or above the initial level. The vasodilatation of the toe persisted unchanged. In the remaining 4 patients who experienced hypotension (upper limit of sensory anesthesia T7, T6, T4, T3), both toes and fingers showed a marked decrease in the size of the pulse waves. In these, the intravenous administration of ephedrine prompted a large increase in size of waves of the toe and a lesser increase in size of pulse waves of the finger as blood pressure was restored. The expected vasodilatation of the toes during spinal anesthesia thus was not manifest until after the restoration of blood pressure.

DISCUSSION

The dilatation of cutaneous blood vessels of the lower extremities is a generally accepted counterpart of spinal anesthesia and is attributed to pharmacologic interruption of the sympathetic nerves carrying vasoconstrictor impulses to these vessels (5). Our findings are in close agreement with this concept. The progressive increase in size of the pulse waves can be accounted for

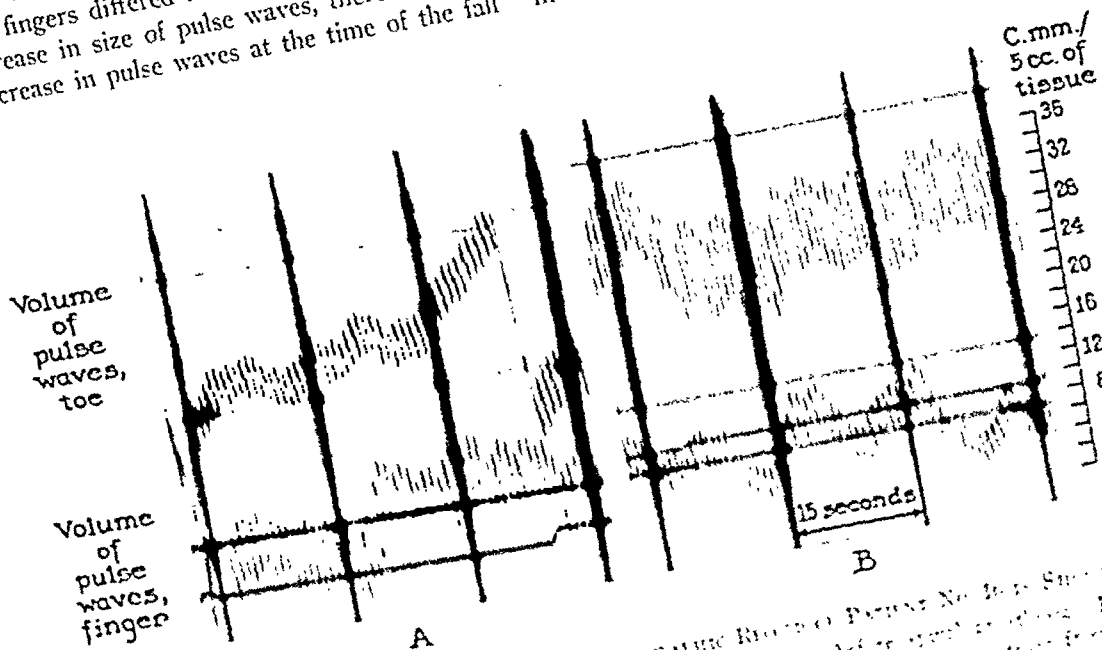


FIG. 1. A PORTION OF THE ORIGINAL PLYTHYSMOGRAPHIC RECORD OF PATIENT NO. 10. (A) SHOWS THE PULSATIONS IN THE SECOND TOE TIP AND INDEX FINGER TIP BEFORE SPINAL ANESTHESIA. (B) SHOWS THE PULSATIONS 3 MINUTES AFTER INTRODUCTION OF THE ANESTHETIC DRUG. THE TOE PULSES INCREASED IN SIZE FROM 7 TO 12 C. MM. WHILE THOSE OF THE FINGER DECREASED FROM 7 TO 4 C. MM.

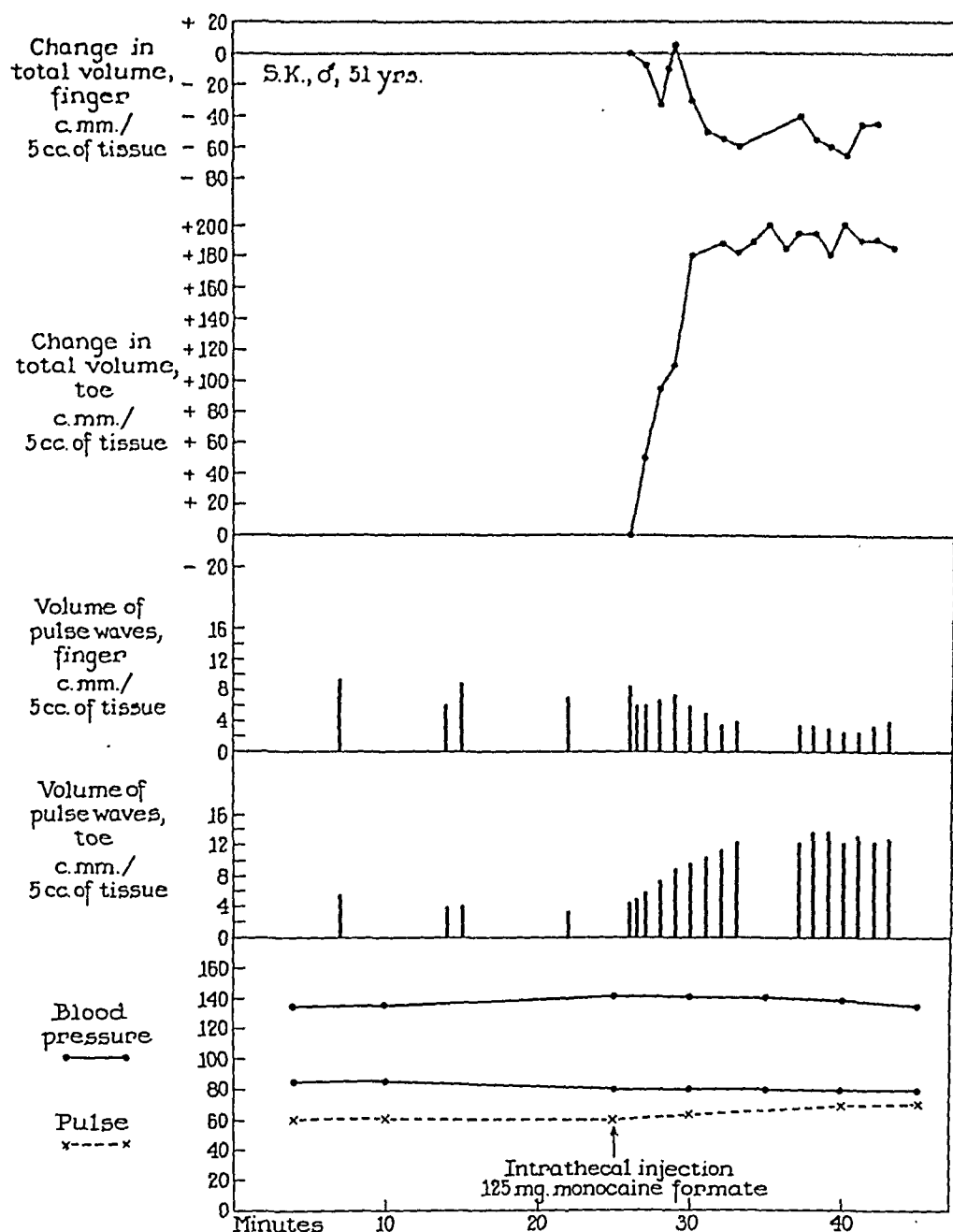


FIG. 2. THE CHART SHOWS: (1) CHANGES IN TOTAL VOLUME OF FINGER AND TOE, AND (2) CHANGES IN VOLUME OF PULSE WAVES OF FINGER AND TOE DURING ONSET OF SPINAL ANESTHESIA IN PATIENT No. 1

Blood pressure and pulse rate remain essentially unchanged. The upper limit of sensory anesthesia reached T8.

on the basis of loss of cutaneous arteriolar tone when the sympathetic nervous supply is interrupted. The decrease in size of the *alpha waves* is explained by postulating interruption of intermittent vasoconstrictor stimuli traversing sympathetic nerves. The production of *alpha waves* is not fully explainable but the present belief is that in the normal subject (in the absence of anesthesia), at irregular intervals of 8 to 15 seconds,

vasoconstrictor impulses traversing the sympathetic nerves reach the small blood vessels lead to two effects, namely, temporary decrease in volume of pulse waves and temporary decrease in volume of the finger or toe (*alpha waves*). Intermittent impulses effect intermittent vasoconstriction; passive vasodilatation follows the resultant pattern is one of rhythmic decrease and increase in the size of the part. The

increase in the total volume of the toe during anesthesia may be accounted for by assuming either that the content of the peripheral vascular bed has increased, or that fluid has passed into the local tissues, or that both have occurred.

The changes which were observed in the fingers during the onset and course of spinal anesthesia may be considered as the opposite of those manifest in the toes. Superficially, there may be cause for disagreement with this statement because the alpha waves decreased in volume in both fingers and toes. In the case of the toes, the explanation lay in an interruption of the intermittent impulses necessary for rhythmic vasoconstriction. In the case of the fingers, the evidence supplied by the onset and persistence of small pulse waves suggests that vasoconstrictor impulses have become so closely spaced as to provide no periodic relaxation of the small blood vessels necessary for the formation of alpha waves.

The compensatory occurrence of vasoconstriction in a distant portion may help explain the ability of the body to maintain a normal blood pressure in spite of the marked vasodilatation of a major portion of the peripheral vascular bed during spinal anesthesia. Only in the patients in whom vasoconstriction of the finger occurred and persisted during spinal anesthesia was blood pressure not significantly lowered. When, for reasons not yet understood, this physiologic adjustment did not occur, hypotension ensued.

There are no data in the present experiments to support the theory that vasoconstriction or vasodilatation of such a small peripheral portion as the toe is actually representative of vasoconstriction or dilatation of the entire lower extremity and lower abdominal viscera. If this were not so, however, it would seem unlikely that enough change in circulatory status could result from dilatation of cutaneous vessels of the legs alone to: (1) make the patient during spinal anesthesia highly susceptible to hemorrhagic shock (6); and (2) lead to vasoconstriction of the small vessels of the finger. Similarly, if vasoconstriction or dilatation of the fingers were not representative of vasoconstriction or dilatation of the upper extremity and upper trunk, it would seem unlikely that vasoconstriction of the finger would so uniformly be present when normal blood pressure is

retained during spinal anesthesia and be absent when hypotension supervenes. The vasoconstriction of the finger during spinal anesthesia is, then, considered to be: (1) representative of vasoconstriction in a more extensive vascular bed, and (2) compensatory for the vasodilatation of an extensive vascular bed including the lower abdominal viscera and lower extremity (7). The exact extent of these larger vascular beds cannot be defined at present.

The mechanisms of the mediation of the vasoconstrictor response of the vascular bed represented by the finger during spinal anesthesia are presumably none other than those which normally operate to support the blood pressure during physiological stress (7). In the special situation occasioned by spinal anesthesia, the peripheral vascular bed of the lower extremities cannot be called into play, and as a consequence, there is a heightened response of those vascular beds whose sympathetic nerve supply is still intact. Therefore, resiliency of circulatory response during trauma and hemorrhage is lost to the patient having spinal anesthesia, not only because the blood vessels of the lower portion of the body are pharmacologically denervated and thus unable to react, but also because those of the upper portion are already in a state of compensatory partial constriction. Thus, when further demands are made upon the circulation, these vessels undergo very little additional constriction.

The results of these studies of peripheral circulatory response to spinal anesthesia suggest that there may be two, more or less separate, mechanisms which produce hypotension. In one group of patients, the hypotension seems to result from a loss of, or failure of development of, a compensatory constriction of the small blood vessels whose sympathetic nerve supply is intact. In these patients, the administration of ephedrine restored the constriction in the intact vessels but left the vessels of the denervated toes unaffected. This is in keeping with the findings of other investigators who demonstrated that ephedrine acts to constrict only those vessels whose sympathetic nervous supply is intact (8). In the patient who showed decrease in size of pulsations of the toes and fingers concomitant with fall in blood pressure, it is probable that a decrease in cardiac output is in part responsible for the hypotension.

(6). With complete interruption of the sympathetic nervous supply to the lower extremity a decrease in size of pulse waves of the toes cannot indicate vasoconstriction but is most likely a passive reflection of a decrease in cardiac output. The mechanism responsible for such a diminution of cardiac output cannot be explained for the present. It is probable that paralysis of the cardiac nerves is not of primary importance in these patients since in two of them the upper limit of anesthesia was at the sixth and seventh thoracic dermatomes. Again the effects of ephedrine in these patients are consistent with those reported by most investigators who noted a marked increase in cardiac output attendant upon the administration of the drug (9).

There is other evidence which supports the supposition that under many normal conditions there is partial constriction of the peripheral vascular bed, the loss of which in one portion of the body is compensated by additional constriction in another position. In patients in whom the stellate ganglion was anesthetized by infiltration of 1½ per cent procaine hydrochloride, plethysmograms were taken simultaneously from the index finger tips of the ipsilateral and contralateral hands (10). The plethysmogram from the ipsilateral finger showed vasodilatation and increase in size of the part while the contralateral finger showed vasoconstriction and decrease in total volume. Conversely, the opposite effect occurs when excessive vasoconstriction is induced in one extremity. Faradic stimulation was applied to the distal portion of the lumbar sympathetic chain after section at the level of the first lumbar ganglion during the course of sympathectomy for the reduction of arterial hypertension. The plethysmogram from the ipsilateral toe showed intense vasoconstriction 3 to 5 seconds after the application of the stimulus and of 30 to 45 seconds' duration. The plethysmogram of the index finger showed vasodilatation synchronous with the vasoconstriction of the toe (11).

Three patients subjected to section of the lumbar and splanchnic sympathetic nerves were examined immediately following operation and as long as 2 years later (11). On each occasion, there was marked vasodilatation of the toes and marked vasoconstriction of the fingers. The vasoconstriction of the fingers was far more in-

tense than that seen at any time before the operation. None of these patients had a good therapeutic response to sympathectomy. Just as in the normal patient under spinal anesthesia, vasoconstriction in the upper extremity is useful in maintaining a normal blood pressure, so it would seem that vasoconstriction of the upper extremity following lumbar and splanchnic sympathectomy may militate against the desired reduction of blood pressure.

SUMMARY

Simultaneous plethysmographic records were obtained from the fingers and toes of 28 patients during the onset and early course of spinal anesthesia. In 20 patients who experienced no fall in blood pressure, the expected vasodilatation of the toes occurred, associated with concomitant vasoconstriction of the fingers which was interpreted as representing a compensating mechanism for the support of the circulation. In 4 of the 8 patients who experienced a fall in blood pressure, vasodilatation of the toes occurred unaccompanied by vasoconstriction of the fingers. Following administration of ephedrine, vasoconstriction developed in the fingers and the blood pressure rose, while the vasodilatation of the toes continued. In the other 4, fall in blood pressure was accompanied by small pulse waves in both toes and fingers, owing, presumably, to decrease in cardiac output. Following administration of ephedrine, the blood pressure rose to or above the initial level, the pulse waves of the toes increased markedly (vasodilatation), while those of the fingers remained small (vasoconstriction). In addition to its other effects, ephedrine acted to restore necessary compensating vasoconstriction to the fingers.

Because vasoconstriction of the fingers seemed necessary for the maintenance of the initial blood pressure during spinal anesthesia, it was considered as an index of vasoconstriction in a larger, but not exactly defined, vascular bed of the upper portion of the body. Similarly, the vasodilatation of the toes was considered as an index of vasodilatation in a large vascular bed of the lower portion of the body, on the basis that vasoconstriction of the fingers could hardly be evoked by vasodilatation of the toes alone. The mechanisms for the mediation of the response of compensating

vasoconstriction were presumed to be none other than those vasomotor reflexes which are in operation normally for the support of blood pressure. The heightened response in the fingers was attributed to the call for increased vasoconstriction wherever possible in the face of loss of ability of the vascular bed of the lower portion of the body to respond.

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THE RELATIONSHIP BETWEEN CONCENTRATION OF SULFAMERAZINE IN BODY FLUIDS AND THE RESPONSE IN TREATMENT OF MENINGOCOCCIC MENINGITIS¹

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Numerous studies of bacteriostatic activity of sulfanilamide-type compounds indicate that for effective action a minimal concentration must be attained and that, in general, rising concentration is accompanied by increased activity. Therapeutic trials in experimental animals have led to a similar conclusion. However, attempts to correlate the response of the patient with the concentration of the sulfonamide in the body fluids have not met with much success. It is not surprising, therefore, that divergence of opinion exists as to the importance of this aspect of sulfonamide therapy. The belief widely held among clinicians and reflected in some reviews (1) is that the concentration of sulfonamide in blood is not of critical significance in treatment of many infections.

However, there is general agreement that concentration of sulfonamide in body fluids is a factor of considerable importance in the treatment of meningococcic meningitis, a view shared by those (1) who question the importance of measurement of drug concentration in other infections. Recent reviews (2, 3) have advocated the maintenance in meningitis therapy of blood levels of 10 to 15 mgm. per 100 ml. for sulfanilamide or sulfadiazine. Lower levels have been found to give satisfactory results when sulfathiazole was used (4).

It is the purpose of the present paper to evaluate the response of patients suffering from meningococcic meningitis to treatment with a highly effective sulfonamide, sulfamerazine (methyl sulfadiazine, 2-sulfanilamido-4-methyl-pyrimidine), with particular reference to the concentration of this

substance in body fluids. The comparative significance of drug concentrations in plasma, plasma ultrafiltrate, and in cerebrospinal fluid also has been examined together with the distribution of sulfamerazine between these fluids. The results make it possible to define more clearly the advantages of high as compared with low concentrations of sulfamerazine in body fluids in treatment of meningitis.

Although there is a possibility that sulfonamide therapy may in time be supplanted by penicillin for treatment of meningitis, the relative merits of the two agents remain to be evaluated. It is believed that the data here presented may be of assistance in making a comparison of the two.

EXPERIMENTAL

Through the cooperation of the staffs of the Medical and the Pediatrics Departments of the Philadelphia General Hospital, it was possible to observe the results of treatment of meningococcic meningitis with sulfamerazine in 188 patients. The observations extended over 18 months, and included periods in the winters of 1942-43 and of 1943-44 during which this disease was epidemic. Observations on 95 patients have already been reported (5), along with details concerning the management of the patients. Patients received intravenously 3 grams of sodium sulfamerazine and 1 gram orally of sulfamerazine on admission to the Fever Ward and subsequently, 1 gram orally of sulfamerazine every 4 hours. The only important departure from our previous methods of treatment has been the use of supplementary alkali therapy during 1943-44 for approximately half of the patients.

Cerebrospinal fluid was obtained by lumbar puncture either without local anesthetic or after infiltration with 1:1000 nupercaine. It was ascertained by test that nupercaine would not give color with the reagents used for determination of sulfamerazine. Many of the data in the literature on concentrations of sulfonamides in cerebrospinal fluid are valueless because of contamination of the fluids collected with procaine. The method of

¹ This work was aided by grants from Sharp and Dohme Laboratories, Glenolden, Pa. and from The Research Fund for Infectious Diseases, University of Pennsylvania, Philadelphia.

Bratton and Marshall (6) adapted to the Klett-Summerson photoelectric colorimeter was used. Ultrafiltrates of serum were prepared at 37.5° C. by the method of Lavietes (7). In this method, plasma or serum is filtered under pressure through a sheet of cellophane. A variable proportion of the sulfonamide, depending largely on the compound used, filters through the cellophane. That remaining is loosely combined with serum protein.

Blood serum or plasma was employed for analyses in preference to whole blood because the unequal distribution of sulfamerazine between cells and plasma introduces an additional variable, the concentration of cells, when whole blood is used. Concentrations of sulfamerazine in whole blood average approximately 0.7 of the plasma concentration (8), but the ratio may vary widely.

RESULTS

Relationship between concentration of sulfamerazine in plasma, plasma ultrafiltrate, and cerebrospinal fluid

The average daily concentration of sulfamerazine in blood serum or plasma and in cerebrospinal fluid simultaneously collected during treatment from a representative section of the group is shown in Figure 1. It will be seen that the concentration in serum approximated 16 mgm. per 100 ml. except for a transient rise during the fourth day. As with other sulfonamides, individual plasma and cerebrospinal fluid sulfamerazine differed widely, despite the fact that all patients received the same amounts. Variability of concentrations shown by each patient, however,

was much less than that between different patients. The close dependence of cerebrospinal fluid on serum concentration is clear. Ratios of the two averaged 0.41. A slight trend toward higher cerebrospinal fluid/plasma ratios as treatment progressed was not significant statistically. The relationship in individual patients between plasma sulfamerazine concentration and that in cerebrospinal fluid is shown in Figure 2. Despite the wide range of plasma concentrations represented, from 1.3 to 39.0 mgm. per 100 ml., the relationship remained the same at high and low levels. Some individuals differed markedly from the general trend, with cerebrospinal fluid/plasma ratios as high as 0.66 and as low as 0.19. The highest ratios were observed in gravely ill patients and were explained by decrease in protein-bound sulfamerazine in plasma. Variations in protein-bound sulfamerazine of patients under these and other circumstances will be reported elsewhere.

Sulfamerazine concentration in cerebrospinal fluid is approximately 80 per cent of the concentration in ultrafiltrates of serum, as Figure 3 shows. If cerebrospinal fluid sulfamerazine concentration depended upon ultrafiltration alone, the values would fall along the diagonal, and departure therefrom represents the effects of the intervention of the tissues constituting the plasma/cerebrospinal fluid barrier. Mainly, however,

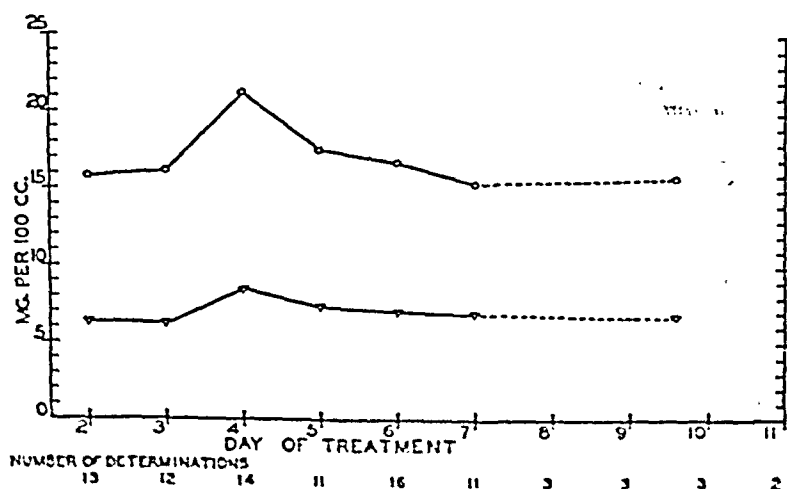


FIG. 1. UPPER CURVE: AVERAGE SERUM SULFAMERAZINE CONCENTRATION DURING THE PERIOD OF TREATMENT. LOWER CURVE: AVERAGE CONCENTRATION OF SULFAMERAZINE IN CEREbroSPINAL FLUID COLLECTED SIMULTANEOUSLY

concentration in cerebrospinal fluid is dependent upon the concentration of drug in plasma, and on such factors as plasma protein concentration and composition. These govern the proportion of the sulfamerazine that is combined with plasma protein and consequently is not ultrafilterable. Comparison of Figures 2 and 3 shows a diminution in scatter in Figure 3 as compared with Figure 2, and presumably much of the variation in cerebrospinal fluid/plasma ratio is due to variations in protein-bound sulfonamide. There was no change in relationship between sulfamerazine concentration in plasma ultrafiltrate and in cerebrospinal fluid in the course of treatment, nor did there appear to be any relationship between concentration of cerebrospinal fluid protein either to the preceding or to sulfamerazine concentration.

Relationship between concentration of sulfamerazine in body fluids and clinical response

The marked individual differences in average concentrations of sulfamerazine in body fluids during treatment apparently influenced the response to therapy. In Figure 4, the time required

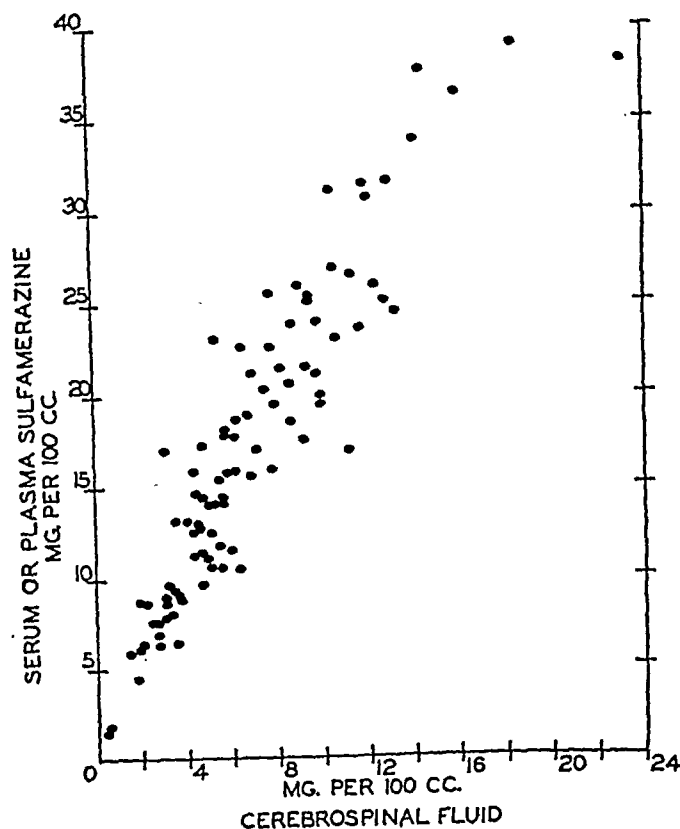


FIG. 2. RELATIONSHIP BETWEEN SERUM AND CEREbroSPINAL FLUID LEVELS

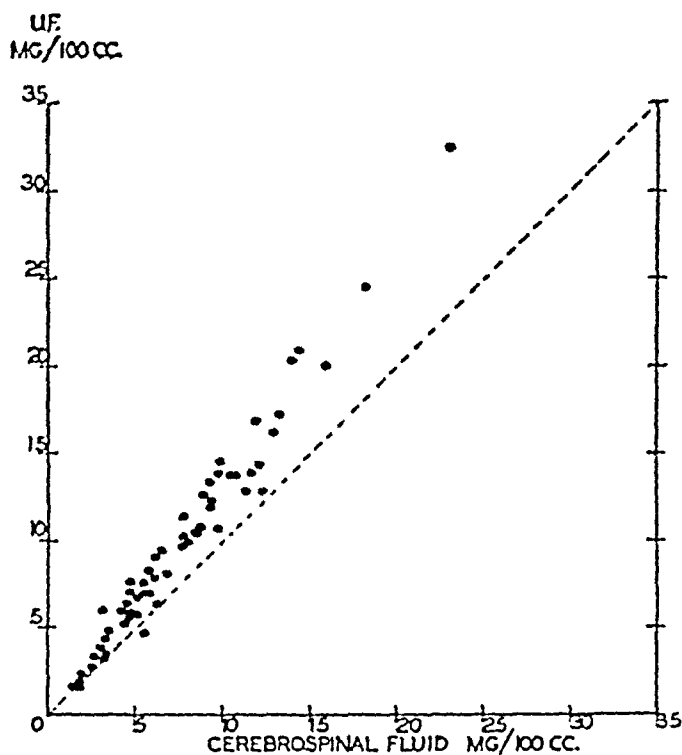


FIG. 3. RELATIONSHIP BETWEEN ULTRAFILTERABLE SULFAMERAZINE OF PLASMA AND CEREbroSPINAL FLUID SULFAMERAZINE

The diagonal represents the relationship to be expected if ultrafiltration alone determined the distribution.

for the patient's temperature (oral) to return to normal is compared with the average sulfamerazine concentration observed in the blood serum. In the presence of higher concentrations of sulfamerazine, the prevailing response was more rapid than that associated with lower concentrations. The relationship is approximated by a straight line.² Although there were a number of patients

² We have not attempted a detailed analysis of the type of curve that will best fit these data. Calculation of regression by the method of least squares yields a statistically valid regression coefficient ($p < 0.01$). The coefficient of correlation ($r = -0.43$). The corresponding relationship between temperature response and cerebrospinal fluid sulfamerazine (Figure 5) also was statistically significant ($p < 0.02$); for the latter, $r = -0.44$. For ultrafiltrates these constants were $p = 0.02$ and $r = -0.54$. All correlation coefficients were statistically significant but the differences were not.

The symbol p refers, throughout this paper, to the evaluation of the probability of chance explaining the occurrence under consideration. When $p = 0.05$ or less, the finding was considered significant. Methods described in Goulden (18) for evaluation of the significance of a regression coefficient were employed.

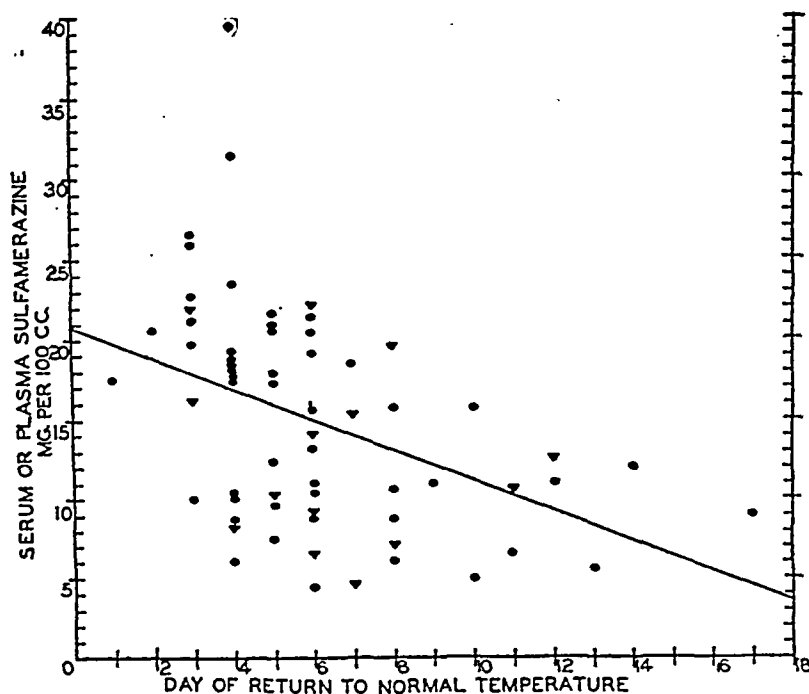


FIG. 4. TIME REQUIRED AFTER STARTING TREATMENT FOR PATIENTS TO BECOME AFEBRILE AS RELATED TO CONCENTRATION OF SULFAMERAZINE IN SERUM OR PLASMA

Triangles: Children under 12. Ovals: All others. Regression line in this and subsequent figures calculated by method of least squares.

with relatively low drug concentrations who responded rapidly to therapy, yet another considerable group showed low concentrations associated with a delayed response. Likewise, most individuals exhibiting high concentrations responded rapidly. Considering the response of the entire group, the average time required for return to normal temperature was shortened one day for each increment of 1 mgm. per 100 ml. in the serum sulfamerazine concentration. No difference is apparent between adults and children under 12, nor between results obtained in 1942-43 and 1943-44. A similar relationship when cerebrospinal fluid sulfamerazine concentration is substituted for that of plasma is shown in Figure 5.

In view of the belief that protein-bound sulfonamides do not possess bacteriostatic activity, it was deemed important to evaluate the correlation between ultrafilterable sulfamerazine and therapeutic action. The relationship shown in Figure 6 resembles closely that of plasma or cerebrospinal fluid. Coefficients of correlation are higher than those for the preceding comparisons but the dif-

ference is not statistically significant. In each instance, high concentrations of sulfamerazine were associated with predominantly more favorable responses than were low concentrations.

Additional evidence relating the rapidity of response to drug level has been derived from evaluation of rate of disappearance of meningitic irritation (Figure 7). Here, the time required for its disappearance is plotted against the average concentration of drug. Again, the patients improving most rapidly were those exhibiting the higher concentrations of plasma sulfamerazine. Calculation of regression gave a statistically highly significant coefficient of regression. The relationship quantitatively is in good agreement with that observed for the response of temperature and that to be described for cerebrospinal fluid protein.

The decline in concentration of cerebrospinal fluid protein³ in the course of treatment with sulfamerazine also shows correlation with the

³ Cerebrospinal fluid protein was determined by electrophoretic turbidimetric method of Lowry and Weig (19).

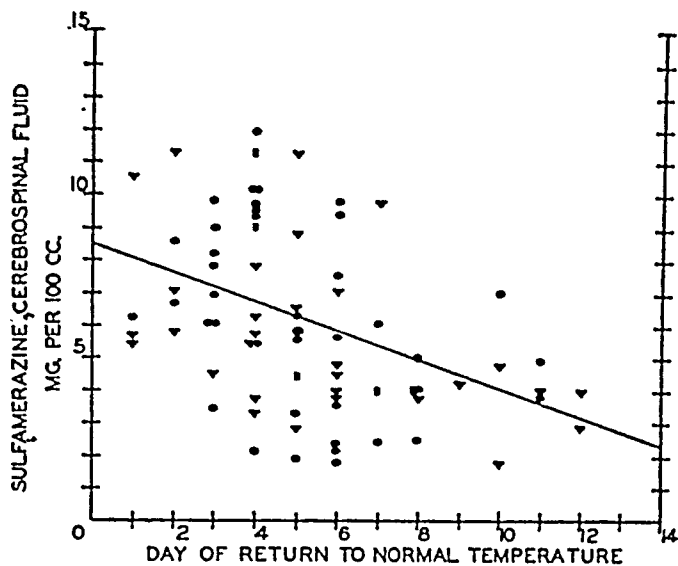


FIG. 5. DURATION OF FEVER AS RELATED TO CONCENTRATION OF SULFAMERAZINE IN CEREBROSPINAL FLUID

Triangles: Patients studied in 1942-43. Ovals: Patients studied in 1943-44. Squares: Samples obtained after temperature became normal.

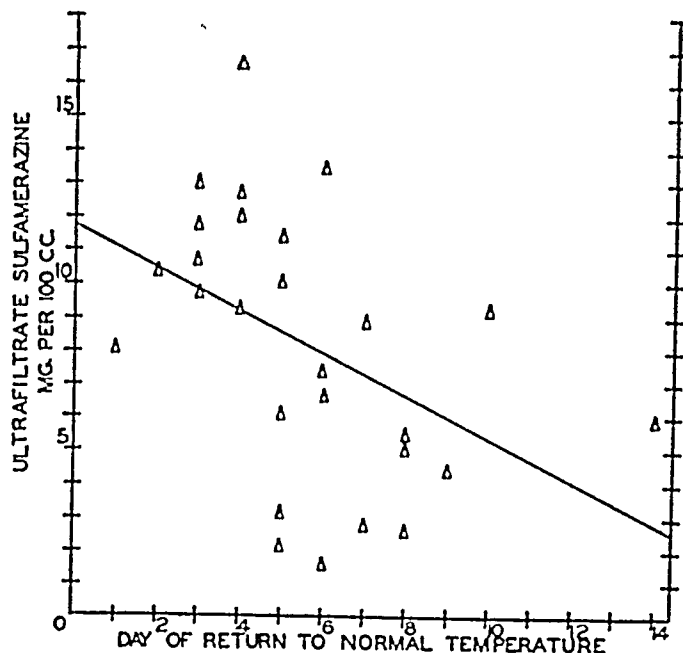


FIG. 6. DURATION OF FEVER AS RELATED TO CONCENTRATION OF SULFAMERAZINE IN PLASMA ULTRAFILTRATE

level of sulfamerazine in plasma (Figure 8). Cerebrospinal fluid samples were collected soon after admission and again as opportunity occurred during treatment. When the concentration of sulfamerazine in plasma was high, the decrease in cerebrospinal fluid protein was more rapid than that occurring in the presence of lower concentrations of sulfamerazine in plasma. This relationship is obscured somewhat by the appreciable time interval between measurements of cerebrospinal fluid protein concentrations. Nevertheless,

less, the relationship, tested statistically, yielded the regression line shown with a regression coefficient that was significant ($P = < 0.02$).

TOXICITY

Forty-six toxic reactions were noted in 40 of the 188 patients. Certain toxic effects of the sulfonamides are thought to be dependent upon the dosage and resulting concentration in body fluids. Since higher concentrations of sulfamerazine appear to be more effective therapeutically

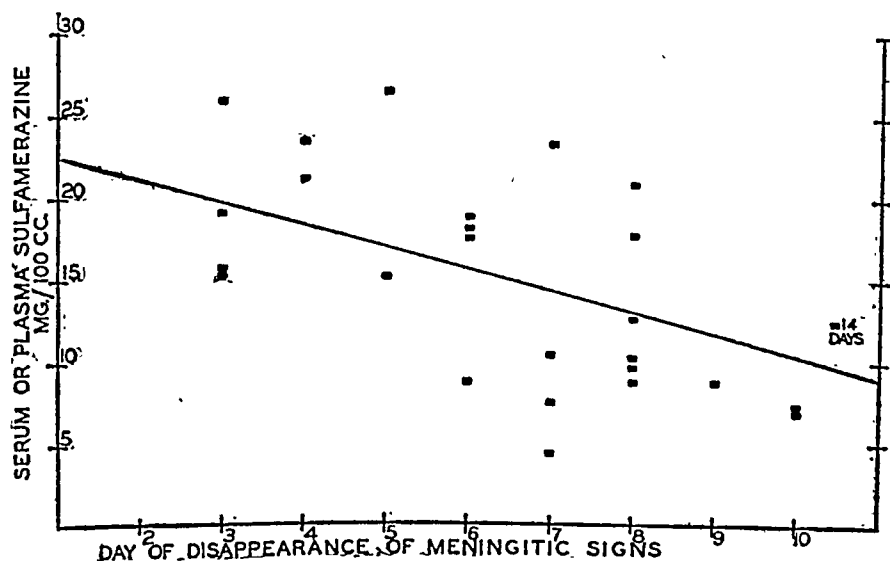


FIG. 7. DURATION OF MENINGEAL IRRITATION AS RELATED TO CONCENTRATION OF SULFAMERAZINE IN SERUM OR PLASMA

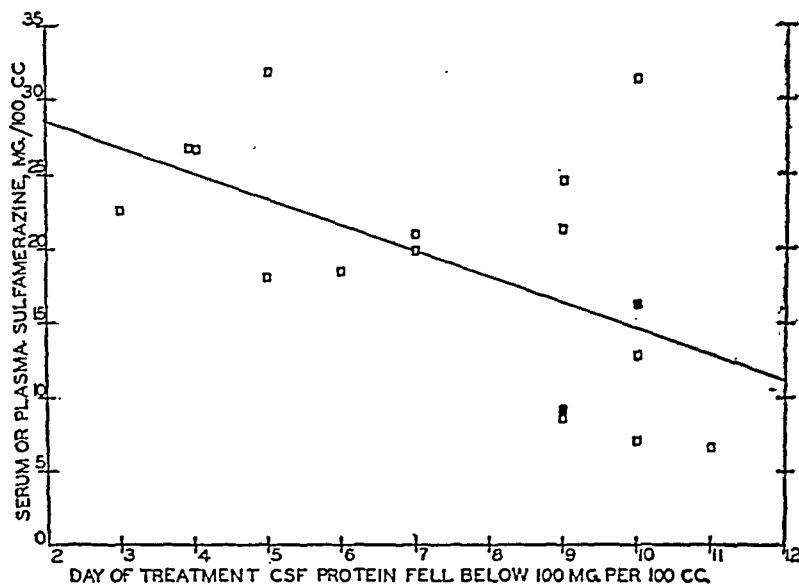


FIG. 8. DECREASE IN CONCENTRATION OF CEREBROSPINAL FLUID PROTEIN AS RELATED TO CONCENTRATION OF SULFAMERAZINE IN SERUM OR PLASMA
Solid squares: Fatal cases; all others recovered.

than lower concentrations, it becomes desirable to evaluate the increased hazard of toxicity from the higher concentrations. Table I shows that such a relationship probably exists in regard to drug fever, since the frequency is much higher in the patients with concentrations in serum above the median level. Statistical analysis indicates⁴ that the relationship approaches the level accepted as significant. Drug rash, on the other hand, shows an incidence well removed from the level of significance, and there is no difference in frequency of hematuria at high and low concentrations in the serum. Certain investigators (9) have stated that renal damage from sulfonamides appeared to occur without any apparent relationship to quantity administered or level in blood.

The higher incidence of toxic effects associated with higher drug concentrations is offset in part by the increased frequency of such reactions as the duration of treatment increases. Figure 9 shows the occurrence of toxic manifestations of sulfamerazine during each day of the treatment period. Since the number of patients undergoing treatment decreased daily, the comparison of early with later periods is best made in terms

⁴ The chi-square method with the Yates adjustment for continuity was employed.

of the proportion of patients undergoing treatment on any day who gave evidence of toxicity. Patients who may have shown more than one toxic reaction are included only once, at the time of the first reaction. The tabulation shows that occurrence of toxic manifestations is clearly dependent upon time and that the frequency rises after 8 days. The distribution appears to be bimodal, a peak occurring at the 5th day and again at the 9th.

TABLE I
Incidence of toxic manifestations at higher and lower concentrations of sulfamerazine in plasma

Toxic manifestation		Plasma sulfamerazine		P
		Below 14.5	Above 14.5	
Microscopic hematuria	Present	6	5	>0.5
	Absent	60	60	
Drug fever	Present	2	9	0.05
	Absent	64	55	
Rash	Present	2	7	>0.15
	Absent	64	58	

† Median: mgm. per 100 ml.

information available for sulfamerazine and sulfadiazine and for meningitic infections, it is not yet possible to correlate with any certainty the figures available for humans with such concentration-response curves. However, they do provide a guide as to the type of relationship to be sought.

Remaining to be considered is the possibility that the correlation we have observed between concentration of sulfamerazine in body fluids and clinical improvement may have been the result of a coincidental alteration of the pharmacodynamic behavior of the drug, resulting perhaps from illness of greater severity affecting the patients exhibiting low sulfamerazine concentrations, or other cause. All patients were receiving the same dosage, yet showed the marked differences in concentrations seen in Figures 2 and 4. Age, incidence of complications, duration of illness, and other factors were comparable for the patients with high and those with low sulfamerazine concentrations. Sulfamerazine is not readily excreted by the kidney and it appears unlikely that differences in rate of excretion are responsible except that certain high values are so explained. Differences in absorption from the gastrointestinal tract thus must be the main cause of the differences in drug concentration. It is conceivable that some common factor such as nutritional deficiency might influence unfavorably both absorption of sulfamerazine and response to infection. That the behavior observed was not due to gradual decrease in concentration of sulfamerazine in the body fluids is shown in Figure 1, which shows that values are well sustained during the period in which the concentration-response relationship was observed. It seems probable, therefore, that differences in concentration of sulfamerazine in body fluids were primarily responsible for enhanced therapeutic effect in patients with high drug concentrations.

SUMMARY

The concentration of sulfamerazine in cerebrospinal fluid is approximately 80 per cent of that of an ultrafiltrate of plasma, and is closely dependent upon the same factors governing the concentration of the drug in the ultrafiltrate, *i.e.*, concentrations of drug and protein in plasma, and composition of the protein.

In treatment of meningococcic meningitis, higher concentrations of sulfamerazine in body fluids were associated with more rapid return of body temperatures to normal, more rapid disappearance of meningitic irritation, and more rapid decrease in cerebrospinal fluid protein concentrations, than were lower concentrations. For each milligram of increase in plasma sulfamerazine concentration, the average response was shortened by one day. The results suggest that the dosage employed and in common use is sub-optimal, and that further trials with therapeutic concentrations in blood serum above 20 mgm. per 100 ml. are needed.

Determinations of concentrations of sulfamerazine in cerebrospinal fluid or plasma ultrafiltrates offer no advantage in evaluation of therapeutic response in meningococcic meningitis over such determinations in plasma.

Certain toxic manifestations (drug fever) of sulfamerazine appear to be more frequent at higher concentrations of drug in plasma. Others (rash, hematuria) showed no such relationship. The incidence of toxic manifestations increased appreciably after one week of treatment, reaching a maximum about 10 days after treatment was started.

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COMPLEMENT FIXATION IN HUMAN MALARIA USING AN ANTIGEN PREPARED FROM THE CHICKEN PARASITE *PLASMODIUM GALLINACEUM*

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The literature concerning various serological reactions in malaria in man has been well reviewed (1). According to this review, attempts to obtain a specific complement fixation reaction with sera from human beings infected by *Plasmodium vivax* or by *Plasmodium falciparum* have led in the past to inconclusive results because no sensitive, specific, and easily standardized antigen was available. Due to the difficulty in obtaining sufficient quantities of heavily infected human blood from which to prepare an antigen, these investigators developed an antigen from *Plasmodium knowlesi* which occurs naturally in the malaria of monkeys and can be used to induce malaria in man. A complement fixation test using such an antigen was found to give a group reaction with sera from human beings infected with *Plasmodium vivax* and *Plasmodium falciparum*.

When the onset of war made procurement of monkeys impracticable, two workers (2) prepared an antigen from the chicken malarial parasite *Plasmodium gallinaceum*. One of them (3) demonstrated the group specificity of this antigen, using the sera of soldiers with malaria at Percy Jones General Hospital. Further studies appeared desirable since there are no certain clinical criteria indicative of a complete cure in malaria and a test that could detect latent malaria would obviously serve a very useful purpose.

The present report deals with the serological investigations conducted at the Harmon General Hospital. The antigen prepared from *Plasmodium gallinaceum* was supplied originally by the aforementioned workers and subsequently by the Army Medical School. The specificity and sensitivity of this complement fixation test was studied

on 11,367 sera. These sera were obtained from 1000 normal healthy soldiers, from 95 soldiers with febrile illnesses other than malaria, from 481 men with syphilis, and from 505 soldiers who gave a history of malarial infection while in the South Pacific. During the period of study of the last-named group, there were 434 recurrent attacks of malaria due to *Plasmodium vivax*.

MATERIALS AND METHODS

Sera. One thousand sera for controls were obtained from normal healthy soldiers. None of these men had been overseas or had a history of malaria. Four hundred and seventy-five sera were obtained from 95 soldiers who likewise had never been overseas or had malaria but who were suffering from a febrile illness in which the body temperature was not less than 100° F. at the time of drawing the initial blood sample. From each of these 95 patients, sera were taken for 5 consecutive days. Sera were taken from 481 men with syphilis who had never been overseas. These men were negro soldiers from various parts of the country and it is possible that some from the South may have had malaria contracted in the past. From the 505 patients with a history of malaria contracted in the South Pacific, sera were obtained at 5-day intervals and, when a recurrent attack of malaria developed, sera were taken on the day of onset and on each of the next 4 days. The taking of sera at 5-day intervals was then resumed.

Smears. A thick or thin blood smear was made from capillary blood each time that serum was obtained by venepuncture. Staining was done by the Giemsa technique. The species of plasmodium was determined in each instance with special reference to the possibility of mixed infections.

Treatment. Acute malarial attacks were treated with atabrine, 2.8 grams in 7 days, for 50 per cent of the attacks; atabrine, 2.8 grams in 7 days followed by atabrine 0.1 gram daily for 60 days, in an additional 31 per cent; quinine, 16 grams in 7 days, in 3 per cent of attacks; quinine, 6.0 grams in 2 days followed by atabrine 1.5 grams in 5 days followed by plasmochin 0.15 gram in 5 days, in 3 per cent; and various other combinations of these drugs in 5 per cent of attacks. The details of treatment for the remaining 8 per cent are not known because the attacks took place while the patients were absent from the hospital.

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² Major, MC, A. U. S.

³ Captain, SnC, A. U. S.

⁴ Colonel, MC, A. U. S.

Complement fixation technique. Patients were bled from an antecubital vein with sterile equipment and the blood placed in sterile tubes. Serum was removed promptly from specimens of blood and kept sterile so that retesting could be carried out at future dates. All stored sera were frozen and kept in this state. For use in the test, the sera were inactivated for 30 minutes at 56° C.

The antigen consisted of lyophilized chicken red blood corpuscles infected with *Plasmodium gallinaceum*. Using the titer stated on the container, the volume was always restored according to directions.

Complement was obtained from the Army Medical School to which it had been furnished by a commercial firm in a lyophilized form, packaged *in vacuo*. The complement was always restored to the volume directed on the ampule. Before use, each lot of complement was titrated to determine its potency by the method given below.

The amboceptor used was obtained from the Army Medical School and its unit was determined by titration.

Fresh sheep red blood corpuscles were obtained as needed. Twenty ml. of citrated blood were obtained twice a week and washed as directed for use in the Kolmer test. The corpuscles were originally used in a 2.5 per cent suspension, but later when the test was revised, a 2 per cent suspension was used. The accuracy of the suspension was tested by centrifuging 10 ml. of the suspension in a graduated centrifuge tube and noting the volume of the packed cells. The suspension was never more than 2 days old at the time of use.

The salt solution originally used was made up to 0.85 per cent and unbuffered. Later the salt solution was buffered with 0.005 M phosphate (pH 7.2 to 7.4).

For a short period of time, the tests were set up according to Coggeshall and Eaton's technique as modified by Captain A. W. Frisch, MC, of Percy Jones General Hospital. Later the test was altered to conform to the Army Medical School procedure (4).

Frisch's technique was as follows:

1. Titration of amboceptor

A. Preliminary titration: Titrations were carried out in the presence of an excess of complement (*i.e.* 0.2 ml. of a 1:10 dilution). For titration purposes, the following dilutions of amboceptor were prepared: 1:100; 1:200; 1:400; 1:800; 1:1600; 1:3200; 1:6400.

To each tube containing 0.5 ml. of diluted amboceptor, 0.2 ml. of 1:10 complement and 0.2 ml. of saline were added. Then to each tube was added 0.25 ml. of 2.5 per cent sheep red corpuscles. The tubes were shaken and incubated in the water bath at 37° C. for 30 minutes and then read. The highest dilution of amboceptor showing complete hemolysis was selected for the final titration.

B. Final titration: Using the dilution of amboceptor, as determined above, the following graded amounts of amboceptor were added to a series of 7 tubes.

Tube	1	2	3	4	5	6	7
Amboceptor—ml.	0.50	0.40	0.30	0.20	0.10	0.05	0.025
Saline—ml.	0.20	0.30	0.40	0.50	0.60	0.65	0.675
Complement 1:10 ml.	0.20	0.20	0.20	0.20	0.20	0.20	0.20
2.5 per cent sheep R.B.C.—ml.	0.25	0.25	0.25	0.25	0.25	0.25	0.25

The tubes were then incubated in the water bath for 30 minutes at 37° C. and read. The smallest amount of amboceptor giving complete hemolysis was taken as 1 unit. In the final test, there were 2 units contained in a volume of 0.25 ml. The amboceptor dilution containing 2 units remained constant for each lot, provided that the red blood corpuscles were prepared properly.

2. Complement titrations

Complement was titrated each day that the tests were run. A series of tubes was set up containing 1:10 complement as follows:

Tube	1	2	3	4	5	6
Complement—ml.	0.20	0.15	0.10	0.075	0.050	0.025
Saline—ml.	0.50	0.55	0.60	0.625	0.650	0.675
Amboceptor (2 units)—ml.	0.25	0.25	0.25	0.25	0.25	0.25
Sheep cells (2.5 per cent)—ml.	0.25	0.25	0.25	0.25	0.25	0.25

The tubes were incubated for 30 minutes at 37° C. and read. The exact unit of complement was taken as the amount showing complete or almost complete hemolysis. For the final test two units of complement were used.

3. Preparation of antigen

The lyophilized antigen was restored to original volume with distilled water and then diluted 1:10 with saline. The antigen was shaken thoroughly and then allowed to stand to get rid of the larger particles. The turbid supernatant fluid was pipetted off and serial dilutions of the antigen from 1:10 through 1:320 were prepared.

A series of two rows of tubes, A and B, containing the following were set up:

Tube	1A	2A	3A	4A	5A	6A
Antigen 0.25 ml.	1:10	1:20	1:40	1:80	1:160	1:320
Complement units	2	2	2	2	2	2
Known pos. serum—ml.	0.2	0.2	0.2	0.2	0.2	0.2

Tube	1B	2B	3B	4B	5B	6B
Antigen 0.25 ml.	1:10	1:20	1:40	1:80	1:160	1:320
Complement units	2	2	2	2	2	2
Saline—ml.	0.2	0.2	0.2	0.2	0.2	0.2

The tubes were incubated in the water bath at 37° C. for 1 hour, after which 0.5 ml. of sensitized sheep red blood corpuscles were added. The tubes were then re-placed in the water bath at 37° C. until the final row of tubes in row "B" showed complete hemolysis. The dilution of antigen showing complete hemolysis and complementary activity was selected as the end point. It was found that the antigen was invariably constant with that given on the container.

The qualitative test proper: To each of two tubes the following were added:

	Tube 1A	Tube 1B
Serum, ml.	0.2	0.2
Complement units	2	2
Antigen, ml.	0.25	
Saline, ml.		0.25

In addition to the above, there were prepared at the same time two control tubes, one containing a known positive serum set up as above and an antigen control containing 0.25 ml. antigen, 2 units of complement, and 0.2 ml. of saline. The tests and controls were incubated at 37° C. for 1 hour and then 0.5 ml. of corpuscles sensitized with amboceptor were added to each tube. The tubes were incubated at 37° C. until the antigen controls were clear. The results were recorded in terms of plus signs, ranging from 4+ to 0—4+ indicating no hemolysis and each increment 25 per cent more hemolysis until complete hemolysis occurred. If no hemolysis occurred in either control tube or the test tube, the result was recorded as anticomplementary.

Quantitative titrations were performed on all sera that had given a 4+ reaction. In addition to repeating the test on these sera, the following dilutions were prepared: 1:5, 1:10, 1:15, 1:20, 1:30, 1:40, 1:60, 1:80, 1:120, and 1:160. Normal, undiluted sera and sera diluted 1:5 and 1:10, were included in these titrations as controls. After the dilutions had been prepared the tests were performed in the same manner as above.

After approximately 3000 tests had been run, a modification in technique was introduced. With this procedure, the 0.85 per cent NaCl was buffered with 0.005 M phosphate (pH 7.2 to 7.4). The reagents were prepared in the same manner but the volume of each was adjusted so that it was contained in 0.2 ml. and the total volume for each test was 1 ml.

After the reagents were added, the tests were shaken and placed in the refrigerator at 5° C. overnight. The next morning there was added to each tube 0.2 ml. of 2 per cent sheep's corpuscle suspension sensitized with 2 units of amboceptor, making a total volume of 0.4 ml. The tubes were incubated at 37° C. for 30 minutes and then read. The activity of the hemolytic system was tested by refrigeration of the controls overnight followed by addition of the indicator series the following morning.

More than 8000 tests were performed using the ice-box fixation method. At one time, because of the larger number of negative tests, it was thought that even though the complement unit was determined by titration, an excess was possibly being used. Therefore, duplicate tests were set up on the same sera using the complement unit as determined by titration for one series and a two-thirds unit of complement in the other series. This gave a larger number of positive results but also increased the number of anticomplementary results. Recently, we had the opportunity of comparing a large number of tests in duplicate using the old method and one recently introduced by Major C. H. Rein and Captain S. Bukantz (5). The method of Rein and Bukantz has a distinct advantage over the one now in use in

that it lends itself readily to standardization. However, in comparing the results of the latter method with the one employed in this laboratory, it was found that there was no significant difference either in sensitivity or specificity in approximately 1500 tests run in duplicate.

RESULTS

A summary of the results of 11,367 complement fixation tests are shown in Table I. Single specimens of sera from 1000 normal healthy soldiers showed 99 per cent to be negative. Tests on 475 sera from 95 soldiers with febrile illnesses other than malaria showed 96 per cent to be negative. Of 481 sera from syphilitic men, 93 per cent were negative. Of 9411 sera from 505 patients with a history of malaria contracted in the South Pacific area, tested during the entire period in which they were under observation in the Harmon General Hospital, 67 per cent were negative, 30 per cent positive, and 3 per cent anticomplementary. A detailed analysis of the material recorded in Table I is presented in the following figures and tables. The specificity and sensitivity of the complement fixation test and its practical application to the problem of malaria is then discussed.

TABLE I

Results of complement fixation tests performed on sera of various controls and patients with history of malaria

Source of	No. pts.	No. tests	Negative		Positive		Anticomplementary	
			num-ber	per cent	num-ber	per cent	num-ber	per cent
I. Control tests								
1. Healthy soldiers	1000	1000	989	99	9	Under 1	2	Under 1
2. Patients with febrile diseases other than malaria	95	475	455	96	14	3	6	1
3. Syphilitic men	481	481	449	93	24	5	8	2
II. Soldiers with history of malaria *	505	9411	6318	67	2777	30	316	3
Total tests		11367						

* All gave a history of malaria contracted in the South Pacific area but not all developed attacks while in Harmon General Hospital.

Serological controls. Sera were obtained from 4 groups of normal healthy soldiers for the control series. None of these men had been out of the United States and none gave a history of

having had malaria. The first and second groups were soldiers from the detachment of an affiliated hospital unit and their sera were taken during field training. The third and fourth groups were infantrymen from whom the blood was drawn just after completion of a final type physical examination. For each member of a given group, the blood was taken on the same day and all tests were run on the day following procurement of sera. Ninety-nine per cent of all the sera were negative. Additional sera were not obtained from the 9 men with positive complement fixation tests or from the 2 men with anticomplementary results.

As a further control, the results of the complement fixation test were determined in soldiers with febrile illnesses other than malaria. Ninety-five patients with a temperature of not less than 100° F. at the time of taking of the first serum also had specimens taken on 4 additional consecutive days. None of these patients had ever been overseas or had ever had malaria. The illnesses from which they suffered at the time of the tests were common respiratory infections, pneumonia, pleurisy, measles, and acute rheumatic fever. Table II shows that 96 per cent of the 475 sera tested were negative. The positive and anticomplementary results, totaling 20 tests, occurred in 14 patients.

The final series of control sera were from 481 syphilitic men. All of these soldiers had proven syphilis and were under treatment. At the time of testing, Wassermann, Kahn, and malaria complement fixation tests were performed on each

serum. The results showed that 318 Wassermann and 359 Kahn tests were positive. Twenty-four malaria complement fixation tests were positive and 8 were anticomplementary. Out of 481 sera from the total group of syphilitics (none of whom had neurosyphilis), 93 per cent of the malaria complement fixation tests were negative, 5 per cent positive, and 2 per cent anticomplementary.

Titre of complement-fixing antibodies. As an indication of the serological sensitivity of the complement fixation test, 1335 strongly positive sera (4+) from malarial patients were further tested in 10 dilutions. The dilutions ranged from 1:5 through 1:160. The results are presented in Figure 1 and indicate that 40 per cent of all tests were positive in a 1:5 dilution, 29 per cent in a 1:10 dilution, 22 per cent in a 1:15 dilution, 13 per cent in a 1:20 dilution, 4 per cent in a 1:30 dilution, 3 per cent in a 1:40 dilution, 2 per cent in a 1:60 dilution, and 1 per cent from 1:80 to 1:160 dilution. Ninety-four per cent did not titre beyond a 1:30 dilution.

Relation of blood smear and complement fixation during 234 recurrent attacks. For the purpose of this analysis, no one was considered clinically to have a relapse unless a positive blood smear and temperature of at least 100° F. were obtained before treatment was instituted. Figure 2 shows that on the first day of 234 recurrent attacks, the percentage of positive smears was 100. By the second day, with treatment, it had dropped to 63 per cent, by the third day to 10 per cent, and by the fourth and fifth days to 1

TABLE II

Complement fixation in 95 patients with febrile illnesses other than malaria (475 tests)

Diagnosis	Number of patients	Serological results during 5 successive days														
		Negative					Positive					Anticomplementary				
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Common respiratory diseases	54	53	52	49	53	54	1	1	5	0	0	0	1	0	1	0
Measles	27	27	27	27	27	27	0	0	0	0	0	0	0	0	0	0
Pneumonia or pleurisy	13	11	11	13	11	12	2	2	0	2	1	0	0	0	0	0
Rheumatic fever	1	0	0	0	0	1	0	0	0	0	0	1	1	1	1	0
Total by days		91	90	89	91	94	3	3	5	2	1	1	2	1	2	0
Grand total	95	455					14					6				

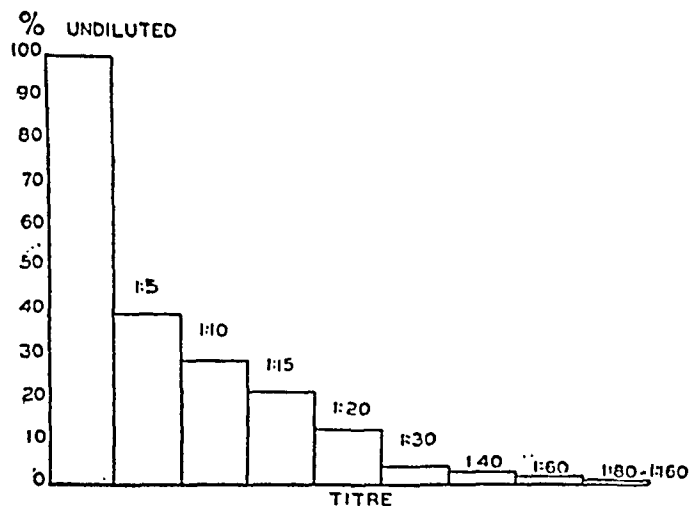


FIG. 1. DILUTION TESTS ON 1335 STRONGLY POSITIVE SERA (4+)

per cent. During the same period of time, the percentage of negative complement fixation tests was recorded (Figure 2) rather than the percentage of positive tests in order to account for the anticomplementary results. It is apparent that, from the first day through the fifth, negative tests were obtained between 41 and 46 per cent of the time. On any one of these 5 days, not over 58 per cent of the tests were positive. Table III shows the daily complement fixation results represented in Figure 2.

Complement fixation during the recurrent attack. Sera were drawn for 5 consecutive days during recurrent attacks beginning at the time a smear was found to be positive. Table IV shows the complement fixation results in 238 attacks

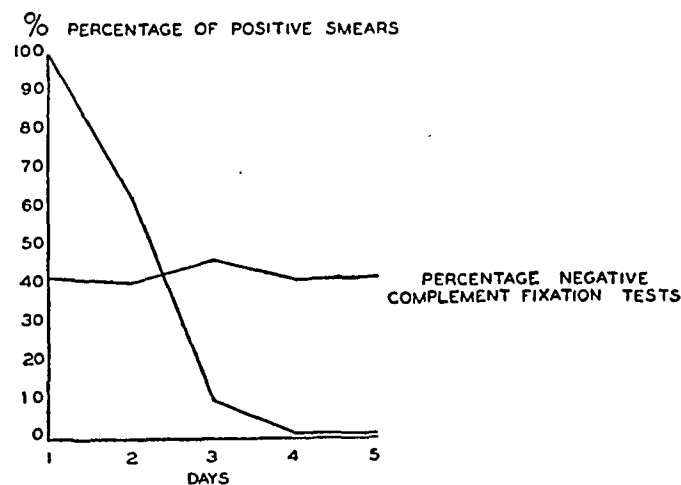


FIG. 2. COMPLEMENT FIXATION AND BLOOD SMEARS FOR 5 CONSECUTIVE DAYS BEGINNING WITH THE FIRST DAY OF A RECURRENT ATTACK (234 ATTACKS)

TABLE III

Daily complement fixation results represented in Figure 2

Days	Results of complement fixation tests						Negative per cent
	0	1+	2+	3+	4+	AC	
1	100	5	19	22	77	11	43
2	96	6	15	19	90	8	41
3	107	6	16	16	71	18	46
4	97	3	17	27	80	10	41
5	100	6	18	20	77	13	43

in which no anticomplementary results were obtained. The results are recorded as negative or positive for each day for each individual attack. The positive results include the entire range from 1+ to 4+. From these data it can be seen that there were 28 different types of serological responses. For example, in 54 attacks not a single positive test was found and in 42 attacks not a single negative test was found. In all the other attacks, there were variations in the days on which either positive or negative results were obtained. These results indicate that a positive

TABLE IV

Complement fixation results for 5 consecutive days in 238 recurrent attacks of malaria

Number in group	Days				
	1	2	3	4	5
54	—	—	—	—	—
4	—	—	—	+	—
1	—	—	+	—	—
9	—	+	—	—	—
5	—	+	+	—	—
3	—	+	+	+	—
1	—	—	+	—	+
2	—	+	—	—	+
1	—	+	+	—	+
1	—	+	—	+	+
10	—	—	—	—	+
8	—	—	—	+	+
8	—	—	+	+	+
13	—	+	+	+	+
3	+	—	+	—	—
3	+	—	—	+	—
1	+	—	+	+	—
12	+	—	—	—	—
8	+	+	—	—	—
4	+	+	+	—	—
12	+	+	+	+	—
3	+	—	—	—	+
3	+	+	—	—	+
5	+	+	+	—	+
5	+	—	—	+	+
8	+	+	—	+	+
9	+	—	+	+	+
42	+	+	+	+	+

TABLE V

Comparison of serological response in 2 successive attacks in 30 patients

Number	First attack Days					Second attack Days					Previous attacks	Interval in weeks
	1	2	3	4	5	1	2	3	4	5		
1	-	-	-	-	-	-	-	-	-	-	2	24
2	-	-	-	-	-	-	-	-	-	-	13	17
3	-	-	-	-	-	-	-	-	-	-	3	14
4	-	-	-	-	-	-	-	-	-	-	6	13
5	-	-	-	-	-	-	-	-	-	+	15	9
6	-	+	-	-	-	+	-	-	-	-	8	20
7	-	+	-	-	+	-	-	-	-	-	2	14
8	-	-	-	-	+	+	-	+	-	-	5	12
9	-	-	+	+	+	-	-	-	-	-	7	23
10	-	+	+	+	+	-	-	-	-	-	2	13
11	-	+	+	+	+	-	-	-	+	+	6	12
12	-	+	+	+	+	+	+	+	+	+	7	4
13	+	-	-	-	-	+	-	+	+	+	8	13
14	+	+	-	-	-	-	-	-	+	-	4	12
15	+	+	-	-	-	-	+	-	-	-	3	13
16	+	+	-	-	-	+	+	+	+	+	8	12
17	+	+	-	-	-	+	+	+	+	+	8	9
18	+	+	+	-	-	+	+	+	+	+	12	11
19	+	+	+	+	-	+	+	-	+	+	4	15
20	+	+	-	+	+	+	-	+	+	+	8	12
21	+	+	-	+	+	+	+	+	+	+	8	7
22	+	-	+	+	+	+	-	-	-	-	9	4
23	+	-	+	+	+	+	+	-	-	+	4	13
24	+	+	+	+	+	-	-	-	-	-	7	15
25	+	+	+	+	+	-	-	-	-	-	8	13
26	+	+	+	+	+	-	-	-	-	+	5	5
27	+	+	+	+	+	+	+	-	-	-	1	5
28	+	+	+	+	+	+	+	+	+	-	5	22
29	+	+	+	+	+	+	-	-	-	+	4	10
30	+	+	+	+	+	+	+	+	+	+	11	16

test might not appear at all during this 5-day period or might appear on any one of the 5 days and might be followed on the next day by a negative test and even later by a positive test. Thus, there was no constant serological response characterizing the attacks as a whole.

Comparison of the serological response in 2 successive attacks in 30 patients is shown in Table V. The number of attacks prior to the first one for which data are presented is given as well as weeks between the 2 successive attacks. There was no correlation between the number of previous attacks and complement fixation results during the attacks. The group of 42 men with positive tests on 5 successive days had an average of 7.0 previous attacks (range 1 to 15), while the group of 54 men with 5 negative results had an average of 7.3 attacks (range 1 to 17). Furthermore, the type of response in each attack was not usually the same.

Relation of complement fixation and blood

TABLE VI

Relation of complement fixation and blood smears during 5 days in 200 recurrent attacks of malaria

Day of relapse	Positive smears Positive C.F.		Positive smears Negative C.F.		Negative smears Positive C.F.		Negative smears Negative C.F.	
	num-ber	per-cent	num-ber	per-cent	num-ber	per-cent	num-ber	per-cent
First	105	53	95	48	0	0	0	0
Second	51	26	67	34	53	27	29	15
Third	8	4	12	6	77	39	103	52
Fourth	2	1	0	0	101	51	97	49
Fifth	2	1	0	0	101	51	97	49

smears during recurrent attacks. In Table VI, data are presented relating the results of complement fixation tests to the malaria smears obtained on each of 5 days in 200 malarial attacks in which treatment with atabrine was started after a positive smear and a temperature of 100° F. were attained. As previously indicated (Figure 2), the percentage of positive smears dropped sharply from 100 per cent before treatment to 60 per cent on the second day, 10 per cent on the third day, and 1 per cent on the 4th and 5th days of the attack. Since the percentage of positive complement fixation tests remained approximately 50 per cent on any day, it is seen that in the third, fourth, and fifth days of the attack, the complement fixation test was positive in 77, 101, and 101 patients, respectively, from whom negative smears were obtained. This might be interpreted as indicating that in those patients in whom treatment had been initiated without obtaining a smear, the complement fixation test might have been used for diagnostic purposes. Actually, in well over 400 recurrences, positive smears were obtained without difficulty during the initial paroxysm of the recurrence which brought the patient under observation; occasionally a positive smear was obtained during a period of prodromal symptoms, occasionally not until the day after the initial paroxysm, or during a second paroxysm. In only 3 patients, however, with symptoms suggesting a malarial recurrence, did smears remain persistently negative.

Complement fixation in intervals between recurrent attacks of malaria. After determining the specificity and sensitivity of the complement fixation test, the most important phase of the entire problem was approached. This is related to the

termining whether the antibodies detectable by this procedure were consistently present between attacks. Should there be persistent antibodies as long as latent or subclinical malaria existed, the test would be of immense practical value in deciding in the individual case whether or not a cure had been established. The data in Tables VII, VIII, and IX answer this question.

TABLE VII

Complement fixation during 100 intervals between observed attacks

Complement fixation	Number tests
Negative	574
1+	32
2+	44
3+	60
4+	151
Anticomplementary	30
Percentage negative: 64	Total 891

Table VII shows the results of complement fixation tests in 100 intervals between 2 or 3 consecutive attacks, observed in 76 patients. The interval chosen was that period of time between the sixth day from the beginning of an attack (since the first 5 days as shown before are arbitrarily considered as the attack) and the last day preceding the next attack. The shortest interval was 4 weeks and the longest 24 weeks with the majority about 8 weeks. Out of a total of 891 tests, 64 per cent were negative and 33 per cent were positive. In other words, only one third of the tests were positive in patients who subsequently developed malaria. It is obvious that the results of initial casual testing during intervals free from clinical activity cannot be used for predicting subsequent recurrences.

Complement fixation directly before and after recurrent attacks. Complement fixation before and after 300 attacks, without reference to consecutive attacks, is shown in Table VIII. Of 171 tests performed 10 days before an attack, 67 per cent were negative. Five days after an attack (10 days from the initial day of the attack) in 223 tests, 48 per cent were negative; ten days after, in 242 tests, 52 per cent were negative; 15 days after, in 224 tests, 59 per cent were negative, and 20 days after, in 215 tests, 66 per cent were negative. It is to be recalled from Figure 2 that during the 5 days of the attack period, 41 per cent to 46 per cent were negative, and from

TABLE VIII

Complement fixation in sera from malarial patients taken 5 and 10 days, respectively, before a recurrent attack and 5, 10, 15, and 20 days after the fifth day of 300 recurrent attacks

Complement fixation	0	1+	2+	3+	4+	AC	Percentage negative
10 days before attack	114	7	9	10	26	5	67
5 days before attack	121	5	8	13	36	6	65
During attack							41 to 46
5 days after attack	103	8	5	16	68	18	48
10 days after attack	125	9	12	14	70	12	52
15 days after attack	133	10	12	25	40	4	59
20 days after attack	142	9	15	11	33	5	66

Table VII, that for the entire interval between 100 attacks, 64 per cent were negative. This shows that the number of positive tests reached a maximum of 58 per cent during an attack and that within 25 days from the onset of an attack there was a drop to 33 per cent positive tests. By chance, 22 tests were made on the day before an attack and 26 tests, 2 days before. Only 14 and 23 per cent, respectively, were positive as compared with 34 per cent 5 days prior to an attack. This finding is not contrary to the theory according to which the test may become negative just before the paroxysm because of absorption of antibodies by the increasing number of parasites. The small number of tests does not permit use of the data either in support or in contradiction of this suggestion.

Complement fixation during a period of 6 months. Table IX represents a continuous serological study covering 6 months' observation at Harmon General Hospital of 121 soldiers known to have had malaria in the South Pacific. Each of these men had a sick furlough of 3 weeks in this 6-month period. The interest in this table lies in the results of complement fixation tests

TABLE IX

Complement fixation results on sera from 121 malarial patients taken at 5-day intervals for 6 months except during the periods of recurrent attacks (5 days)

Serology								
Number patients	Number of attacks in 6 months	0	1+	2+	3+	4+	AC	Percentage negative
1	4	14		2		3	1	70
17	3	210	11	17	25	64	23	60
42	2	580	41	45	56	164	15	64
35	1	602	32	32	39	123	11	72
26	No attack	539	13	24	22	63	5	81

performed at 5-day intervals, exclusive of the periods of recurrent attacks (5 days). The purpose of the compilation is to demonstrate persistence or disappearance of positive complement fixation in patients with recurrent attacks and in those in whom subsequent attacks did not occur during this period of observation and serological investigation. One patient had 4 attacks but during the symptom-free intervals had 70 per cent negative tests. Seventeen patients had 3 attacks with 60 per cent negative tests in the intervals. Forty-two patients with 2 attacks had 64 per cent negative tests. Thirty-five patients with 1 attack had 72 per cent negative tests. Twenty-six patients with no attacks while in the hospital had 81 per cent negative tests. During their period of observation, these 26 patients had no definite clinical attacks of malaria or parasitemia. Of these, 1 had an attack of unobserved illness, which was probably malaria, 5 at no time complained of symptoms suggesting malaria, and 20 complained irregularly of varying aches or malaise which might have represented subclinical activity. Since, for the most part, these men resided in rehabilitation barracks where routine taking of temperature was purposely omitted, data were not collected which would permit a precise estimate of the relation of occasional rises in complement fixation (19 per cent positive tests) to subclinical activity. If the tests of the first 3 months of the 6-month period are separated from those of the last 3 months, the percentage of negative tests for the latter period is found to be 86 per cent.

DISCUSSION

As long ago as 1907 DeBlassi (6) claimed to have secured positive specific reactions in human malaria with an antigen prepared from *Plasmodium vivax*. The idea of utilizing a group reaction was introduced by Coggeshall and Eaton who demonstrated that an antigen from *Plasmodium knoelesi* could be used in studies of human malaria. Subsequently Kligler and Yoeli (7) using an antigen from *Plasmodium gallinaceum* found that it was nearly as effective as that attained with *Plasmodium knoelesi*.

In our investigations, comparison of the number of positive tests in control sera with those

of patients known to have had malaria showed that a group reaction could be obtained in sera from patients infected by *Plasmodium vivax* when an antigen prepared from *Plasmodium gallinaceum* was used. False positive tests reached as high a figure as 5 per cent in the group of syphilitic men studied, whereas it was under 1 per cent in normal healthy men. When the results on sera from the syphilitics are grouped with our other control data, the specificity of this test for malaria appears to be somewhat less than that of the various complement fixation tests for syphilis (8), although obviously the procedures are not strictly comparable.

The sensitivity of the malaria complement fixation test was determined by dilution tests on strongly positive sera. At a dilution of 1:5, only 40 per cent of the sera were still positive, and at a dilution of 1:30, only 4 per cent were positive. It is impossible to compare these dilution tests accurately with complement fixation tests for syphilis because of differences in the character of the diseases and in the types of antigens. Nevertheless, it is of some interest that in dilution tests performed on 275 of the positive Wassermann tests in our control series of 481 syphilitic men, 55 per cent were positive at a dilution of 1:60, 33 per cent at 1:120, and 3 per cent at 1:600.

The results of the malaria complement fixation test during the 5-day period of the attacks were variable. It was possible for an individual to have negative tests throughout or in contrast to have positive tests on each of the 5 days. The number of previous attacks had no relationship to the subsequent serologic findings during a future attack. Comparison of 2 successive attacks showed that the results in each were frequently quite different and that the interval between attacks had no relation to the complement fixation results during the attack.

In the interval between attacks, the test could not be used to predict that a recurrent attack would eventually appear or that a patient was cured. In a group of 300 attacks, it was found that the maximum number of positive tests occurred during the attack and that it decreased from 58 per cent to 33 per cent within 24 days. Five days prior to an attack, the percentage of positive tests was 34. In a very small series of

22 tests on the day before an attack and in 26 tests two days before an attack, only 14 and 23 per cent, respectively, were positive.

Over a 6-month period, the number of prior attacks and the total length of infection had no appreciable effect on the number of positive tests.

Whether the antibodies of immunity are those detected by this test is not known. The decline in percentage of positive tests following the attacks may conceivably be related to a fall or disappearance of sufficient available antibodies. This might then reflect the clinical evidence of lack of immunity as indicated by subsequent relapses. Such an impression would suggest that the *plasmodia* reside in reservoirs during the intervals between attacks at which time the circulating antibodies decline quantitatively, although some may be present at all times. However, it is also possible that the antibodies utilized in this test have no relation to the problem of immunity in this disease.

In syphilis, a positive complement fixation test is usually obtained while the patient is still actively infected and this may cover a period of years but in the type of malaria studied in this investigation, an individual removed from an endemic area may still be actively infected as shown by subsequent relapses and yet intermittently have negative tests.

Successful application of a complement fixation test in recurrent malaria depends both upon the specific properties of the antigen employed and the patient's antibody response to the disease. Factors which may have influenced the antibody response of these patients during the period in which these tests were performed were: (1) previous residence in endemic zones under more or less continuous suppressive treatment; (2) previous occurrence in most of the patients of multiple attacks of malaria due to infection with *Plasmodium vivax* and/or *Plasmodium falciparum*, with possible difference in strains within these species; (3) variation in length of time from original infection to the time of serological examination, with tendency toward a lower rate of attack with the passage of time; and (4) prompt initiation of therapy during the attacks under study with resultant disappearance of trophozoites from the blood.

SUMMARY

1. Complement fixation tests using an antigen prepared from the chicken parasite *Plasmodium gallinaceum* were performed on 11,367 sera.
2. Control sera obtained from 1576 men included:
 - (1) Sera from 1000 healthy soldiers, who had never had malaria. These were negative in 99 per cent of the tests.
 - (2) Sera obtained on 5 successive days from 95 soldiers with febrile illnesses other than malaria. These were negative in 96 per cent of the tests.
 - (3) Sera from 481 syphilitic men without a history of malaria. These were negative in 93 per cent of the tests.
3. Sera were obtained from 505 soldiers evacuated from the South Pacific and known to have had malaria while in that area. The results on these sera showed that:
 - (1) From this group, 9411 sera were positive in 30 per cent of the tests.
 - (2) There was a maximum of 58 per cent positive tests on any one of 5 successive days during 234 recurrent attacks.
 - (3) Complement fixation tests before and after 300 recurrent attacks showed that:
 - a. Five days before the attacks, 33 per cent of the tests were positive; 5 days after the attacks, 47 per cent were positive; and 20 days after the attacks, 33 per cent were positive.
 - (4) In 121 patients followed for 6 months at 5-day intervals, 95 had recurrent attacks during the period of study, whereas, the remaining 26 had no attacks during that period. The sera from all of these men, exclusive of the period of attacks, showed that only 36 per cent of the tests were positive.
4. These results indicate that the complement fixation test using an antigen prepared from *Plasmodium gallinaceum* gives a group reaction of undetermined sensitivity for the sera of human beings infected by *Plasmodium vivax*, but is of no practical value in detecting latent malaria or indicating when a patient is cured.

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STUDIES ON THE QUANTITATIVE EVALUATION OF CERTAIN TREATMENTS IN THE HEALING OF EXPERIMENTAL THIRD DEGREE BURNS¹

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The number of local treatments which have been proposed for burns is legion. In one exhaustive review of the subject (1), some sixty pages are devoted to this phase. It is obvious, however, that there is in the surgical mind little satisfaction with these treatments because, at not infrequent intervals, medications continue to be suggested for topical application to burned surfaces. It was one such report (2) which prompted the investigations to be presented below.

Although attempts at quantification of wound healing and the effects upon it of certain treatments have been made in the past (3 to 7), to our knowledge such attempts have never been applied to an evaluation of treatment of the third degree burn including its excision. From all accounts, expedition of healing of the third degree burn presents a major challenge to therapy. Excision of such an area has been performed at numerous times in the past (1). It was perhaps first performed in 1901 (8). In 1913, two workers (9) believed that by such a procedure survival of the burned animal could be prolonged. It was decided, therefore, to investigate systematically the healing of the experimental third degree burn and the effect thereon of several treatments including excision, and to apply to the data obtained the newer knowledge of growth quantification (10).

METHODS

Adult rabbits weighing between 5 and 9 pounds were used. Standard third degree burns were made on the backs of rabbits according to the technique of Leach *et al.* (11), using an asbestos-jacketed cylinder through which hot water circulates. The burning surface of the cylinder was a circle one inch in diameter, and the burning temperature was 75 to 82° C. for 1 minute.

There were 3 general types of experimental procedure carried out following the burn. The first of these was upon a group of 20 burns in which no treatment was administered. The second group consisted of 54 cases in which some medicament or graft was applied immediately following the burn; in 13 of these, the application was made directly on the burn, and in the other 41, to the surface left following excision of the burn. The excision line was usually through the area of edema just beyond the coagulum. Despite this conservative approach, the retraction of the wound usually led to an area double that of the third degree burn. Finally, the third group consisted of 5 donor areas (Table I).

The coagulated plasma-sulfonamide film of Pollock (2), hereinafter known as CPS, was applied in 13 cases to the burned area, and in 15 cases to the surface remaining after the burn had been excised.

Other reagents applied to the surfaces left by excision of the burn included vaseline (13 cases), thrombin-fibrinogen-sulfonamide (5 cases), and a "diazofilm" (1 case).

Grafts were attempted in 7 cases but did not succeed for various reasons, chief among which was sepsis. Both whole and split-thickness grafts on both whole and split-thickness excision surfaces were tried.

Bandaging septic excisions led to early gross infection, maceration, and destruction of tissue with a punched out appearance, but improvement occurred almost immediately upon exposure to air. Cultures were not attempted. In sterile experiments, the bandages were rarely left on after 14 to 16 days. Within this time, infection did not occur. Thus, medication was never in apposition to the wound longer than this time.

Observations on the course of regeneration were made approximately every third day from the time the burn was made to the time of complete regeneration. The measurement of the *unhealed* area was made with a centimeter ruler. The surfaces were regarded as roughly elliptical in shape, whence the area was taken to be

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$\pi r_1 r_2$, where r_1 and r_2 are, respectively, the semi-major and semi-minor axes of the ellipse.

The measurement of the unhealed area mentioned above can be converted into a suitable growth measurement as follows: Let $a(o)$ denote the original area of the wound surface. Let $a(t)$ denote the remaining unhealed area after t days. Then obviously $a(o) - a(t)$ represents the amount of tissue regenerated. The Carrel-Hartman (3) observation that, in equal times, the growth increment of a large wound is greater than that of a small wound is very likely attributable to the use of just $a(o) - a(t)$ as the parameter of growth. This measure can give the impression that the fundamental proliferation mechanisms proceed at different rates in wounds of different sizes. As a matter of fact, the "percentage growth rates" are the same regardless of size. It is preferable therefore to put large and small wounds on an even footing, so to speak, and use instead of amount regenerated, the

$$\left\{ \begin{array}{l} \text{Fraction of the original} \\ \text{wound which has been} \\ \text{regenerated up to time } t \end{array} \right\} = \frac{a(o) - a(t)}{a(o)} = P$$

Assuming that area is proportional to some function of cell numbers, P also represents the fraction of the total number of cells which have been produced to time t . In terms of this parameter, then, differences among growth curves will arise only from fundamental differences in the mechanism of growth, rather than from mere size difference of the wounds.

From the experimental data for each regeneration, it is possible to compute P at every experimental point. For days other than days of observation, one may obtain an approximation to P by a linear interpolation. Thus, it becomes possible to plot "mean growth curves" for large numbers of areas by plotting average values of P at a given time. The mean curves thus obtained are remarkably regular, and very well suited for comparative purposes.

RESULTS AND DISCUSSION

Two comparisons of the mean curves for controls with excised areas, and areas treated with vaseline with areas treated with CPS have been

TABLE I.

This table summarizes all experiments considered in the text.

The upper number of each entry is A , the per cent rate of growth, while the lower number is proportional to B , the interactive coefficient. Every entry represents about eight observations of one growth curve. Burning time is given in minutes, and burning temperature in degrees Centigrade. Abbreviations are as follows: CPS = coagulated plasma-sulfonamide film; Thr.-Fibr. = thrombin-fibrinogen.

Experiments in which burn was not excised					Experiments in which burn was excised						
Animal	Burn time and temp.	Untreated burns (control animals)	Burn + CPS	Means of non-excised areas	Excised + vaseline	Excised + unsuccessful graft	Excised + CPS	Excised + Thr.-Fibr.	Excised + S or diazifilm	Donor areas	Mean of excised
2	1-75	0.3337 0.0834		0.3337 0.0834		0.3255 0.0459	0.7110 0.1580			0.3428 0.0365	0.4598 0.0801
3	1-75					0.2290 0.0472	0.3438 0.0844			0.2845 0.0390	0.2858 0.0535
4	1-75	0.2492 0.0238		0.2492 0.0238		0.1867 0.0148	0.3197 0.0524			0.2964 0.0337	0.2676 0.0333
7	1-75		0.6035 0.0457	0.6035 0.0457		0.2897 0.0181	0.6035 0.0457			0.1483 0.0156	0.3472 0.0297
8	1-75						0.1268 0.0063	0.3320 0.0105	0.3530 0.0318		0.2706 0.0459
10	1-75	0.2643 0.0281	0.8658 0.0651	0.3100 0.0466	0.2700 0.0200			0.4386 0.0457			0.3323 0.0328
11	1-75	0.1769 0.0249	0.1815 0.0154	0.1540 0.0222	0.4852 0.0594			0.1815 0.0154			0.3340 0.0424
12	1-82	0.2632 0.0371	0.2138 0.0301	0.2385 0.0336	0.3778 0.0331			0.2478 0.0182			0.2422 0.0256
13	1-79	0.1860 0.0532		0.1860 0.0532			0.1624 0.0166			0.4259 0.0474	0.2272 0.0410
15	1-78	0.1902 0.0452	0.2462 0.0347	0.2182 0.0518		0.3194 0.0410					0.3253 0.0415

TABLE I—Continued

Experiments in which burn was not excised					Experiments in which burn was excised						
Animal	Burn time and temp.	Untreated burns (control animals)	Burn + CPS	Means of non-excised areas	Excised + vaseline	Excised + unsuccessful graft	Excised + CPS	Excised + Thr.-Fibr.	Excised + S or diazofilm	Donor areas	Mean of excised
16	1-78		0.3160 0.0479	0.3160 0.0479		0.1335 0.0152					0.1335 0.0152
17	1-82	0.1920 0.0315	0.2962 0.0338	0.2441 0.0326	0.3879 0.0269		0.3753 0.0232				0.3816 0.0250
18	1-82	0.2897 0.0742	0.3713 0.0742	0.3300 0.0742	0.2111 0.0169		0.3073 0.0240				0.2592 0.0204
19	1-80	0.2965 0.0582	0.2273 0.0394	0.2619 0.0438	0.1903 0.0134		0.2170 0.0143				0.2037 0.0138
20	1-80	0.1335 0.0243	0.1707 0.0265	0.1521 0.0254	0.2145 0.0101		0.2090 0.0108				0.2102 0.0104
21	1-80	0.2187 0.0370	0.1541 0.0270	0.1864 0.0320	0.3020 0.0213		0.3050 0.0354				0.3030 0.0286
22	1-80	0.1989 0.0301	0.1834 0.0171	0.1911 0.0236	0.3387 0.0204				0.2383 0.0161		0.2885 0.0182
24	1-80					0.3970 0.0269					0.3970 0.0269
27	1-81	0.2395 0.0406	0.1080 0.0204	0.1737 0.0305	0.6040 0.0300		0.4020 0.0490				0.5030 0.0390
28	1-81	0.1645 0.0257	0.1382 0.0216	0.1504 0.0237	0.2620 0.0136		0.3100 0.0131				0.2865 0.0134
30	1-81	0.2390 0.0328	0.1865 0.0338	0.2130 0.0332	0.4310 0.0194		0.2210 0.0095				0.3258 0.0144
31	1-81	0.2780 0.0421	0.2490 0.0377	0.2630 0.0399	0.1800 0.0132		0.2830 0.0130				0.2320 0.0131
Total numbers		20	13	19	13	7	15	4	2	5	22

made (Figures 1 and 2, respectively). Each circle on the graphs represents the mean of a stated number (N) of observations. The smooth curves are theoretical curves to be discussed presently. From these comparisons two important inferences are made: (1) that healing proceeds significantly faster when the burned area is excised, and (2) that CPS has no effect on healing which differs from the effect of an inert reagent, such as vaseline. In all instances, it will be observed that the curves are typical simple growth curves, symmetric about $P = 50$ per cent and showing no discontinuities. To further document these remarks now requires a second step in the analysis of the data.

The contrast between the general properties of two growths can be brought into sharper focus

by an elementary theoretical treatment. This is particularly true when the growth of a more or less homogeneous tissue is considered, for then the growth curve can be linked to certain basic factors in cell economy. Thus, if, as has been done (10), we postulate that the

$$\left\{ \begin{array}{l} \text{Rate of} \\ \text{growth} \\ \text{of cells} \end{array} \right\} = \left\{ \begin{array}{l} \text{Intrinsic proliferation} \\ \text{rate of non-inter-} \\ \text{acting cells} \end{array} \right\} - \left\{ \begin{array}{l} \text{Effect of inter-} \\ \text{actions among} \\ \text{cells} \end{array} \right\},$$

we may form a simple theory (12) to account for the growth of a homogeneous colony so that:

$$\left\{ \begin{array}{l} \text{Intrinsic proliferation} \\ \text{rate of non-interacting} \\ \text{cells} \end{array} \right\} = (\text{constant}) \times N = AN$$

$$\left\{ \begin{array}{l} \text{Effect of} \\ \text{interactions} \\ \text{among cells} \end{array} \right\} = - \left\{ \begin{array}{l} \text{Effect of nutri-} \\ \text{tional and excre-} \\ \text{tional interactions} \end{array} \right\} - \left\{ \begin{array}{l} \text{Effect of} \\ \text{spatial} \\ \text{interactions} \end{array} \right\}$$

$$= -BN^2 - CN^3$$

where N is cell number, and A , B , and C are so-called vital coefficients (13). Putting these together we obtain the differential equations of the growth as,

$$\frac{dN}{dt} = AN - (BN^2 + CN^3) \quad (1)$$

Although the factor C is usually small, it introduces considerable mathematical difficulty (12) into the application of equation (1), and furthermore requires a greater number of experimental points than is here furnished.

However, the simpler form of (1)—omitting the C —originally proposed by Robertson (14), though with a different interpretation (autocatalysis), can be easily applied to give comparative estimates regarding A and B . The integral

form of (1) can be written as,

$$\frac{N}{N(\infty)} = \frac{1}{1 + Ke^{-At}}, \quad \text{and} \quad B = \frac{A}{N(\infty)} \quad (2)$$

As remarked above, $N/N(\infty) = (a(o) - a(t))/a(o)$. Substituting into (2) and rearranging, we have

$$\log \frac{a(t)}{a(o) - a(t)} = -At + \log K, \quad (3)$$

from which it follows that A , the per cent rate of growth of non-interacting cells, can be obtained as the negative of the slope of a $\log_e (a(t)/(a(o) - a(t)))$ vs. t . Then B , the coefficient of retardation due to interactions such as competition or possibly infection can be found from (2), at least up to a constant of proportionality.

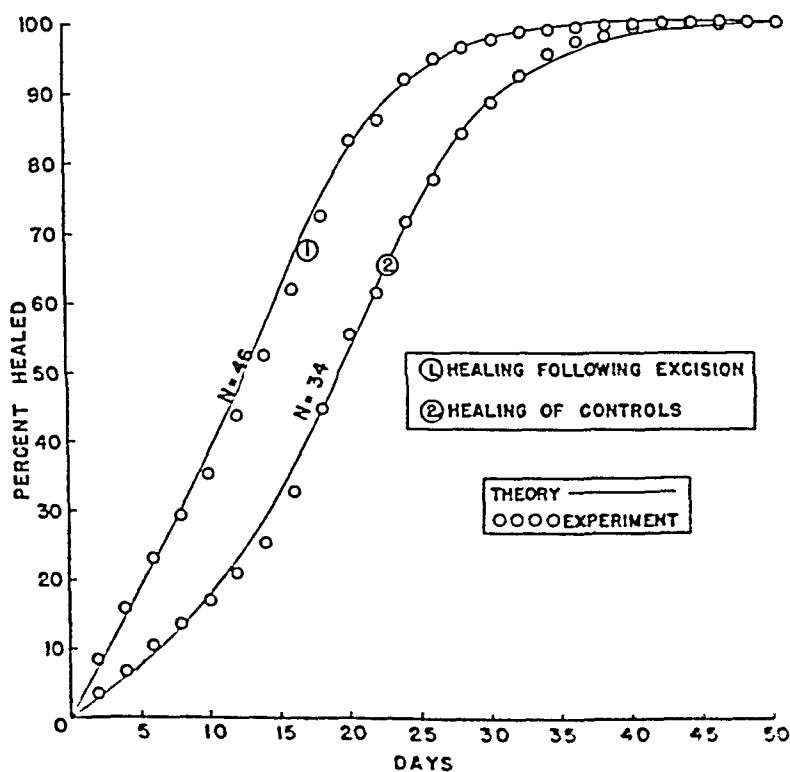


FIG. 1. COMPARISON OF THE EFFECT OF EXCISION (CURVE-1) WITH NON-EXCISION (CURVE-2)

Each circle is the mean of the specified number (N) of experiments. The theoretical curves are, respectively, $P = 100/(1 + 70e^{-1.4t})$, and $P = 100/(1 + 192e^{-1.1t})$, and are derived from the theory of growth of homogeneous cell populations (see text).

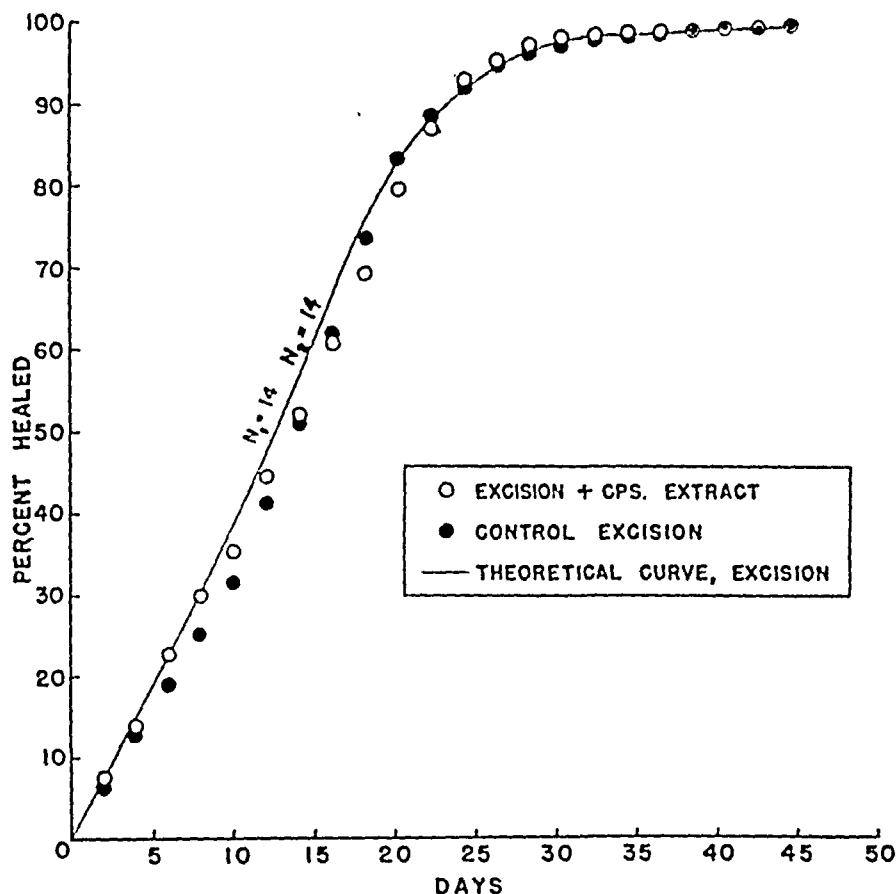


FIG. 2. ILLUSTRATION OF THE FAILURE OF CPS IN AFFECTING THE CURVE OF HEALING

Each white and black point is the mean of 14 paired observations. The control excisions were surfaces coated with vaseline. The theoretical curve is the mean theoretical curve for the healing of any excision surface, $P = 100 / (1 + 70e^{-0.198t})$.

Equation (2) has been applied and A and B have been determined in each of 79 cases with encouraging results. A typical plot of equation (3) appears in Figure 3. In the great majority of cases, the equation was followed with precision, from which fact it can be inferred that the *healing phenomenon, or perhaps the limiting process of the phenomenon, closely resembles the growth of a more or less homogeneous cell population*. Such an over-enthusiastic interpretation of this result can be easily challenged on histological grounds. For example, the contraction of a collagen network may be largely responsible for the closing of a wound, and this process in turn may be paced by the migration of fibroblasts from a regenerating blood supply. The possibility remains, however, that either or both of these agencies may be regarded as homogeneous populations of units whose function is contributing

directly to the closure of the wound rim, and hence obeying the law of equation (2). Obviously, the present experiments cannot give the final answer to this question; yet it would be unwise to overlook the suggestions which arise from them. Quite aside from the question of mechanism, it should be emphasized that the constants A and B, as theoretically determined, measure respectively a per cent rate of growth and an inhibitory interaction amongst the growing tissue. They are mathematical consequences of the experimental data, and are as real as the data.

The evaluation of these constants (Table I) has been applied to yield certain interesting results such as the frequency distributions of A and B for various experimental conditions (Figure 4).

In the comparison of excised burns with control burns, it appears that no statistically significant difference exists between the proliferation rates

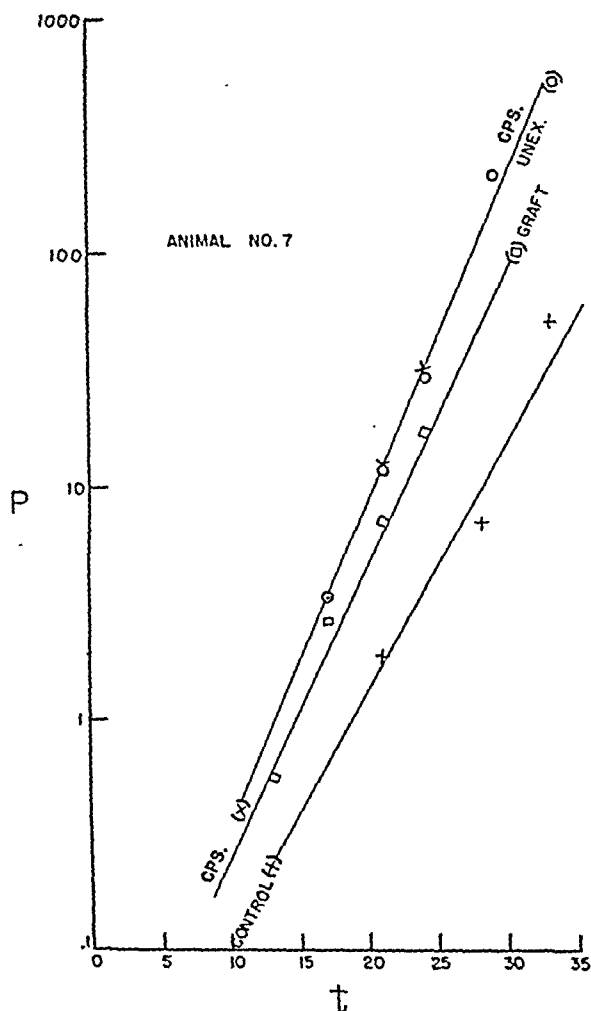


FIG. 3. A TYPICAL PLOT (ANIMAL NO. 7) OF $\text{Log } [a(t)/(a(o) - a(t))]$ vs. t

Actually the values of the argument of the logarithm have been plotted on a semilogarithmic grid. Tangents of the angles of inclination can be multiplied by 0.1382 to yield logarithmic slopes to the base e .

(A's) in the two cases, but there is only one chance in 500 ($t = 3.16$; $P < 2$ per cent) that the difference in the average B's for the two groups will be quite accidental. Thus, keeping in mind the precautions discussed above, we may say tentatively that the average per cent proliferation rate following an unexcised burn is not significantly lower than the rate following excision, but that on the other hand, the inhibitory interactions are far greater when the burn is not excised. The failure to detect any change in the per cent proliferation rate does not support the

concept that the burned tissue releases some growth-depressing substance.

The other frequency distributions (Figure 4) further emphasize the fact that the CPS has no effect either on A or on B when applied directly to the burn, or following excision. It is quite possible that the favorable observations made on this extract were on other than third degree burns. The determination of the degree of the burn in the present investigation was unequivocal.

A glance at the growth constants for the other reagents, e.g., sulfonamide, diazofilm, or graft-donor (Table I), will show that within our data no difference can be said to exist between them and the constants for the vaseline-treated wounds.

SUMMARY

1. The rates of epithelization of experimental third degree burns treated by various methods have been determined and analyzed according to newer knowledge of growth formulas.

2. The superiority of a coagulated plasma-sulfonamide film as a therapeutic agent in the treatment of third degree burns over other agents has not been substantiated in this investigation.

3. Surgical excision of a small third degree burn in rabbits significantly shortens its healing time. The main effect of this treatment seems to be in the removal of inhibitory cell interactions rather than on the proliferation rate.

4. Vaseline and sulfanilamide, thrombin-fibrinogen, and plasma sheets applied to these surgical wounds seem to have approximately the same effect on regrowth as does no medication.

5. The curve of healing of both control and excised burns follows the growth curve of a homogeneous cell colony with high precision.

Lieutenant (jg) C. Martin Rhode, MC-V(S), USNR, had completed experimental work of this report before leaving for service with the Amphibious Forces. He had also left a clear outline of the interpretation of the work. From this and from numerous discussions with him, we have tried to set forth his findings and opinions in a suitable manner. However, responsibility for any of the shortcomings in the present report must rest with us (M. F. M., E. L. L.).

For very helpful statistical and pathological counsel, we are indebted respectively to Lieutenant (jg) J. J. Birren, MC-V(S), USNR, and Lieutenant (jg) J. H. H-V(S), USNR.

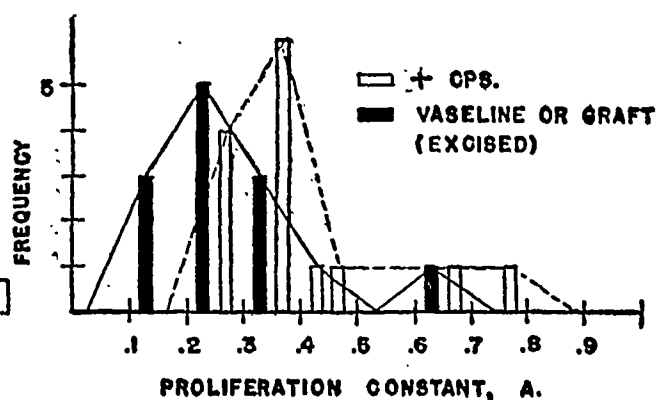
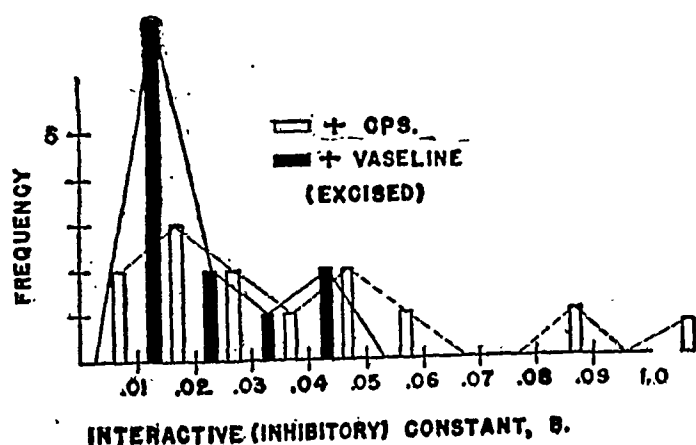
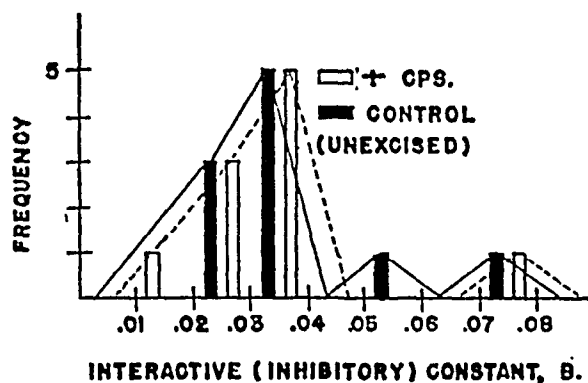
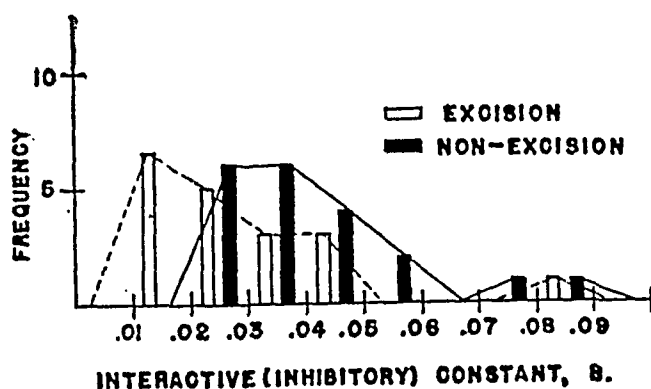
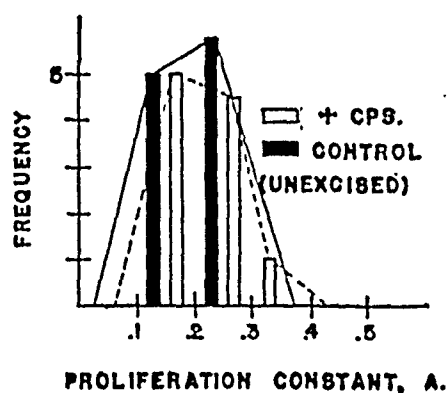
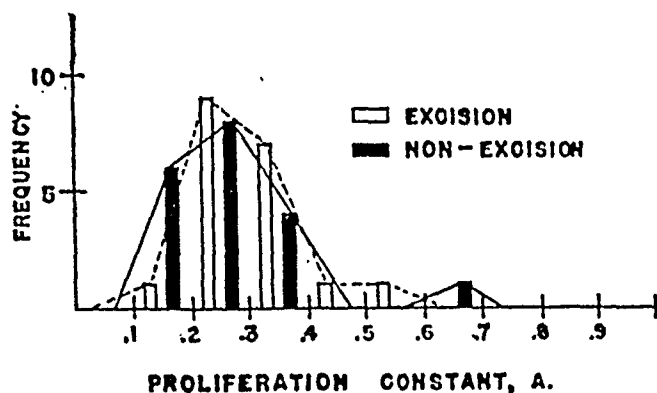


FIG. 4. HISTOGRAMS ILLUSTRATING THE DISTRIBUTION OF GROWTH CONSTANTS A (PER CENT RATE OF PROLIFERATION), AND B (INTERACTIVE CONSTANT) IN THE COURSE OF CERTAIN EXPERIMENTAL COMPARISONS. No distributions are significantly different except those for B in the comparison, excision *vs.* non-excision.

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THE EFFECT OF AMINO ACIDS ON SERUM AND URINE CREATINE

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There is now unequivocal evidence that creatine is formed from glycine, arginine, and methionine (1). It is equally well established that creatine can be transformed into creatinine and excreted in this form by the kidneys (2). Creatine is also concerned with the intermediary metabolism of carbohydrate.

By a photometric modification of Folin's method, devised by Peters (3), Tierney and Peters (4) demonstrated that the serum of normal men contained measurably significant amounts of creatine. They found that the serum creatine was elevated in patients with hyperthyroidism. Creatinuria was almost invariably associated with concentrations of serum creatine greater than 0.6 mgm. per cent, suggesting that creatine, presumably filtered in the renal glomeruli, is completely reabsorbed in the tubules when its concentration in the serum is less than 0.6 mgm. per cent.

The present paper deals with the serum creatine and creatine excretion in various pathological conditions and after certain induced physiological disturbances.

METHODS

Patients on the wards of the New Haven Hospital were studied. Serum from blood collected under oil and centrifuged with anaerobic precautions was analyzed by the method described by Peters (3). Specimens of urine were collected whenever possible during the course of the experiments and analyzed by a similar method, using the suggestion of Tierney and Peters (4) that the urine be diluted to a specific gravity of 1.010. It was found that if 5 ml. of urine, already diluted to a specific gravity of 1.010, were further diluted to 200 ml., the concentration of creatine more regularly fell into a range suitable for photometric measurement.

All experiments were conducted in the morning, after patients had been without food for 10 hours or more. No food was given until the experiments were completed, except in the 2 instances in which the effects of large meals were observed. For the glucose studies, 50 ml. of 50 per cent glucose were injected intravenously. In other experiments, 50 grams of an enzymatic hydrolysate of casein (Amigen, made by Mead, Johnson and

Co.) were injected intravenously, either as a 10 per cent solution or as a 5 per cent solution in 5 per cent glucose. This contains 6 grams of nitrogen. Blood samples were collected before the injection, 5 minutes after the termination of the infusion, and at intervals thereafter. Samples were never taken from the vein used for the infusion. All determinations were made in duplicate and, if these did not check, were repeated or discarded. Changes of serum creatine smaller than 0.2 mgm. per cent are not considered significant.

RESULTS

In Table I are observations on 26 men and 13 women with various diseases. Of the men, only 7 had creatinuria, and in all of these, serum creatine exceeded 0.7 mgm. per cent. SP had no creatinuria during the experiment although his serum creatine was 1.62 mgm. per cent. Creatine did, however, appear in his urine later in the day. This unusual sequence may have been related to renal failure which Rabe (5) has found may disturb the relations between urine and serum creatine in bizarre ways. Women had creatinuria more frequently than men, but only when the serum creatine exceeded 0.5 mgm. per cent. No creatine was found in the urine of MP with a serum creatine of 0.7 mgm. per cent. It is to be noted that on several occasions no significant amount of creatine could be demonstrated in the serum.

With the exception of JCo, all the men with elevated serum creatine were desperately ill; in fact all of them died within 5 days of the observations. This was not true of the women, although the prognosis in the cases of RF, GM, and DB was considered poor or hopeless. This is merely another illustration of the greater tendency to creatinuria among women.

Table II shows the effects on serum and urine creatine of a meal, intravenous glucose, and intravenous casein hydrolysate. Neither the meals nor the glucose infusions altered serum creatine. In the experiment in which urine was obtained

TABLE I

Urine and serum creatine in miscellaneous diseases

Subject	Creatine		Diagnosis
	Serum	Urine ¹	
Male	mgm. per cent	mgm. per cent	
JCo	0.8	27	Fractures of ribs and legs
HC	0.2	0	Regional enteritis
PO	0.7	0	Carcinoma of stomach
HM	0.3	0	Lymphosarcoma
EM	0.1	0	Peptic ulcer with obstruction
CL	0.2	0	Reticulum cell sarcoma
CV	0.1	0	Pernicious anemia
EC	0.2	0	Pernicious anemia
FC	0.3	0	Carcinoma of lung w/ metastases
AS	0.5	0	Carcinoma of lung w/ metastases, jaundiced
ED	0.2	0	Postoperative appendectomy
RS	0	0	Postoperative appendectomy
VP	0.3	0	Postoperative appendectomy
HD	0.3	0	Osteomyelitis of jaw
AG	0.3	0	Empyema
FD	0.1	0	Meningococcus meningitis
JSa	0.1	0	Advanced pulmonary tuberculosis
JCa	1.4	44	Far advanced pulmonary tuberculosis, died ²
TE	1.2	16	Tuberculosis with tuberculous meningitis, died ²
LP	0	0	Undiagnosed muscular disease, ? dermatomyositis
GC	0.2	0	Myasthenia gravis, treated
SP	1.6	0 ³	Bronchopneumonia, died ²
JSi	2.0	46	Bronchopneumonia, died ²
CP	2.6	117	Subacute bacterial endocarditis, died ²
FL	1.4	53	Syphilis, subarachnoid hemorrhage, died ²
BB	1.3	23	Regional enteritis, died ²
Female			
AH	0	0	Pernicious anemia of pregnancy
FH	0.5	0	Diabetes, psychoneurosis
HF	0.5	0	Diabetes, gangrene of foot
MJ	0.3	0	Diabetes, post-acidosis
LD	0.3	0	Acute rheumatic fever with carditis and failure
AR	0.6	29	Acute rheumatic fever with carditis and failure
ET	0.5	8	Heart disease with fibrillation and failure
MF	1.2	8	Hypertensive cardiovascular disease and failure
MP	0.9	19	Ulcerative colitis
DB	0.7	0	
DB	0.9	25	Cerebral thromboses
RF	0.6	11	Chronic febrile disease, ? lupus
GM	0.9	84	Disseminated lupus
AC	1.0	7	Acute trichiniasis

¹ Concentration in urine diluted to 0.010.² All patients who died did so within 5 days of study.³ NPN elevated to 57.

before and after glucose (SP), the rate of creatine excretion did not change.

The response to intravenous casein hydrolysate depended on the initial concentration of creatine

in the serum. In the 4 patients with normal values before infusion, serum creatine remained within normal limits after infusion, although its concentration varied slightly. These variations

TABLE II

The effects of various procedures upon urine and serum creatine

Hours are counted from the end of the infusions or meals. *Start* indicates the initial post-absorptive value. The infusions of casein hydrolysate were given over periods of 2 hours or more. Consequently, values are given not only at the start, but also at the end of the infusion. Urine figures in parentheses represent concentrations of creatine in terms of mgm. per cent, when the rate of urine excretion could not be determined because the period over which it was formed is unknown. Clearances are estimated only over periods in which the concentration of creatine in the serum remained constant (post-absorptive specimens) or when it was descending. In the latter case, it was calculated by the method described by Winkler and Parra (7).

Subject	Time	Creatine		
		Serum	Urine	Clearance
	hours	mgm. per cent	mgm. per hour	ml. per min.
Infusions of casein hydrolysate				
CP	Start	2.3	86	63
	End	2.7		
	1	2.6	40	
	2	2.3		
	3	2.2	120	
GM	4	2.2		
	5		157	120
	Start	0.9	(30)	
	End	1.5		
	1	1.1	74	
DB	2	0.9		
	3	0.8	37	65
	4	0.7	10	24
	5			
	Start	0.9	22	44
JS	End	1.5		
	2	1.1	43	
	4	1.0	29	47
	Start	2.0	(83)	
	End	3.4	(125)	
SP	1	3.4	14	7
	4			
	6			
	Start	1.6	0	0
	End	2.3	0	
BB	2	2.3		
	4	1.0	26	
	6		26	43
	Start	1.3	15	21
	During	1.5	35	
BB	End	1.5	13	
	2	1.0		
	5		4	

TABLE II—Continued

Subject	Time hours	Creatine		
		Serum mgm. per cent	Urine mgm. per hour	Clearance ml. per min.

Infusions of casein hydrolysate—Continued

RF	Start	0.6	(13)	0
	End	0.6		
	2	0.4	10	
	4	0.3	0	
JCo	Start	0.8	40	83
	End	0.6	36	
	2	0.6		105
	4	0.6	39	
VP	Start	0.3	0	0
	End	0.2	0	
	2	0	0	
	4	0		
GC	Start	0.2	0	0
	End	0.2	0	
	2	0	0	
	4	0		
LP	Start	0	0	0
	End	0.2		
	2	0.2	0	
	4	0	0	
RS	Start	0	0	0
	End	0.1		
	2	0	0	
	4	0	0	

Infusions of glucose

SP	Start	1.0	26	
	1/2	1.0		
	2	1.1	26	
JCa	Start	1.5		
	1/2	1.5		
	2	1.5		
JS	Start	3.4		
	1/2	3.4		
	2	3.2		
TE	Start	1.2		
	1/2	1.2		

Regular meal

AS	Start	0.5	0	
	1	0.6		
	3	0.4	0	
MP	Start	0.7	0	
	1	0.8		
	3	0.8	0	

may have resulted from the infusions, or may represent spontaneous fluctuations. On the other hand, in all the patients who had marked elevation of serum creatine initially, the creatine rose significantly after the casein hydrolysate, the peak occurring at the end of the infusion in all cases. The urinary excretion of creatine also rose and fell, usually lagging somewhat behind the changes in the serum. RF and JCo behaved differently from the others. In both, the concentrations of serum creatine, initially in the borderline range in which there may or may not be creatinuria, fell significantly after the infusions, in RF to the point where creatinuria ceased.

Initial and final creatine clearances were measured in 5 cases. In 3 instances (CP, JCo, and GM), they rose significantly, in 1 instance (DB) equivocally. The results, in general, confirm the observation of Tierney and Peters (4), that creatine clearances vary with the concentration of creatine in the serum.

Five duplicate analyses of the casein hydrolysate revealed no significant amounts of preformed creatine.

DISCUSSION

Of the subjects studied, 3 had no measurable amounts of creatine in their sera and 4 others (CV, EM, JSa, and FD) had traces only. This suggests that a zero value by this technique is not necessarily abnormal.

The lack of significant change in the serum creatine following a meal may be due to various factors. The carbohydrate may be without immediate effect, the amount of preformed creatine may be too small to affect the serum appreciably, the amino acids provided by the meal may not be utilized to an appreciable extent for synthesis of creatine. It is possible that creatine is formed, but is so rapidly distributed and utilized that its concentration in the serum does not rise. The acute disturbance of carbohydrate metabolism induced by intravenous injection of 25 grams of glucose evidently has no gross effect upon the general metabolism of creatine.

Since the casein hydrolysate contained no preformed creatine, the elevations of serum creatine after injections of this mixture signify that the formation of creatine increased. They cannot be attributed to failure of excretion because urine

creatinine either increased or remained constant. Presumably the increments were derived from the 3 per cent of methionine and 5.5 per cent of arginine in the hydrolysates. Bloch and Schoenheimer (1) and Borsook and Dubnoff (6) have proved that these amino acids are the materials from which creatine is formed. It is possible that, in the 6 patients who did not develop hypercreatinemia and creatinuria, the synthesis of creatine was relatively inactive, that serum creatine rose in the others because of some particular demand for this compound or because the materials required for its synthesis were not diverted to other purposes.

It is more probable that the synthesis of creatine was equally active in all patients; but that its distribution, utilization, and disposal were retarded in those patients who had initially high serum creatine. The reactions to the amino acids in these experiments are quite comparable to the reactions to ingested creatine reported by Tierney and Peters (4). In the latter, synthesis can have played no part. The falls of serum creatine in JCo and RF may have denoted further acceleration of the processes by which creatine is removed from the blood. The low initial concentrations are merely evidences of the same phenomenon.

The experiments as a whole support the evidence of Tierney and Peters (4) that creatinuria denotes that serum creatine is greater than 0.5 mgm. per cent. In no case of this series was creatinuria encountered when serum creatine was below 0.5 mgm. per cent; in only one was it absent when serum creatine exceeded 0.8 mgm. per cent. The presence or absence of creatinuria in patients with serum concentrations between 0.5 and 0.8 mgm. per cent may be a mark of indi-

vidual variability and technical errors, which may amount to 0.15 mgm. per cent.

SUMMARY

Creatinuria in persons suffering from a variety of diseases was found to be regularly associated with elevated serum creatine.

Serum creatine was not altered by the intravenous injection of 25 grams of glucose or by a large breakfast.

The serum creatine of subjects with initially high serum creatine rose and creatinuria appeared or increased after the intravenous injection of an enzymatic hydrolysate of casein.

The author wishes to express his gratitude to Dr. John P. Peters whose suggestions and criticisms have been most valuable.

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COMPARISON OF THE DIURETIC ACTION OF SODIUM DEHYDROCHOLATE AND MERCUPURIN IN MAN¹

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This paper deals with the results of experiments planned to investigate the diuretic action of sodium dehydrocholate (decholin sodium²) in cardiac patients with congestive heart failure and to compare its action with that of the organic mercurial, mercupurin. While there are several reports in the literature suggesting that decholin sodium has diuretic properties, the methods employed for its study heretofore leave its efficacy open to question. In the present study, the observations were made on ambulatory patients, and the diuresis was determined by change in weight in a manner similar to that described in a previous investigation (1). In this method, the patient loses weight as the result of the dose of the diuretic agent, and regains it by the time of the next injection. It is therefore possible to repeat the same dose, under essentially similar conditions of edema in the same patient, as many times as is necessary to secure a reliable average response to a given dose.

Neubauer (2) appears to have been the first to direct attention to the possible use of decholin sodium as a diuretic agent. He observed that this action in man is feeble and inconstant. Several reports have since appeared describing the diuretic activity of decholin sodium. It did not seem profitable to present a detailed review of all of them, but we may refer to a number of representative papers, sufficient to indicate the statements concerning the diuretic action of decholin sodium which one is likely to encounter. From the scientific standpoint, the literature on this subject is not satisfactory. Many of the reports are based on routine experiences rather than on the results of experiments so designed as to eliminate common sources of error and to

supply data suitable for objective analysis. While there is general agreement that decholin sodium exerts a diuretic action, views differ concerning its extent and regularity. Some found that it rarely fails to produce diuresis (3), others that it fails in 40 per cent of the cases (4). Most reports found it less effective than the organic mercurials (5), others as effective as, or often even more effective than the organic mercurials (6). Certain workers (7) noted that while the daily increase in urine is slight or inconstant, especially in those without edema, the drug regularly increases the urine in the first 2 hours, an effect which subsides in 3 to 5 hours. The view was expressed in some of the earlier papers that decholin sodium prolongs or enhances the diuretic effect of the organic mercurials. It has been stated (8), but without sufficient evidence, that the 2 gram dose of decholin sodium produced the effect of the 1 ml. rather than of the 2 ml. dose of salyrgan, and that the combination of the two agents sometimes caused additive, sometimes potentiative effects. The synergism was stressed in a more recent report (9) but again without evidence, since the paper shows insufficient controls with comparable doses of the organic mercurial alone. The dose employed in most of the reports was 2 grams of decholin sodium given intravenously as 10 ml. of a 20 per cent solution, although doses of 4 grams have also been used (3). It was repeated daily or every other day without apparent poisoning. Most reports noted the bitter taste after the injection. Some observed slowing of the heart rate and temporary fall of the blood pressure by as much as 30 mm. Hg (4), while others observed no effect on either. An occasional rise of the temperature and, after repeated daily injections, a mild diarrhea have been reported (10). It is stated that the drug exerts a diuretic action in normal subjects and in

¹ This study was supported in part by a grant from Riedel-de Haen, Inc.

² Riedel-de Haen, Inc.

patients with edema in association with cardiac, renal, or hepatic disease, the effect being smallest in the normal and greatest in those with cardiac edema (11). Although the effect of decholin sodium is less than that of the organic mercurials, it has been suggested, but without sufficient proof, that it might be useful in cardiac edema not only when other diuretics cannot be used, but when others fail (11).

Little is known concerning the mechanism of the diuretic action of decholin sodium. Observations have been presented (12) in support of the belief that the effect is mediated through a primary action on the mechanism of water storage in the liver. Certain authors (13) noted that intravenous doses of decholin sodium exert a nephrotoxic action in cats and dogs resulting in tubular degeneration. Another (14) was unable to confirm it in cats. The belief has been expressed (15) that both renal and hepatic action are responsible for the diuresis.

EXPERIMENTAL

The observations of the present study were made in a group of 32 ambulatory cardiac patients selected from among 1500 patients in attendance in the cardiac clinics. The characteristics of those that received decholin are summarized in Table I. They all had advanced organic heart disease and far advanced failure with congestion. The more common etiological varieties of heart diseases are represented. All had fairly marked enlargement of the liver due to chronic passive congestion. These patients had been in attendance in our clinics for periods of several years. They were under continuous treatment with digitalis. The heart failure had reached a stage in which digitalis in full dosage was no longer sufficient to maintain the patients in reasonable comfort and free of edema. They were able to remain up and about and to carry on moderate activity only through the aid of mercurial diuretics which had been used in these cases for periods up to 2 years.

The effect of the drug was studied by means of the following: (1) amount of diuresis, (2) size of liver, (3) heart rate, (4) blood pressure, (5) urinalysis, (6) blood N.P.N., (7) subjective symptoms and reactions. The patient was weighed just before the injection, and again, 15 hours later. The loss in weight was taken to represent the diuretic effect. This procedure was repeated every week. For comparison of different drugs or doses, each was alternated with the other for several weeks. Changes in liver size were determined weekly by measuring the perpendicular distance between the tip of the xiphoid process and the liver edge. The heart rate and blood pressure were taken during each visit. The urine was examined for albumin and formed ele-

TABLE I
Characteristics of patients used in study

Name	Age	Sex	Diagnosis*
Ma Sp	75	M	A.S., Hyper., E.H., Cor. Thromb., N.S.R.
Na Sa	58	M	A.S., Hyper., E.H., Scler. Aorta, N.S.R.
Lu He	34	F	R.F., E.H., M.S., M.I., N.S.R.
Le Jo	40	M	L., E.H., A.I., N.S.R.
An Pe	63	M	L., Hyper., E.H., A.I., A.F.
An Wa	67	F	A.S., Hyper., E.H., N.S.R.
Si Ro	73	M	A.S., Cor. Scler., N.S.R.
El Vo	70	F	A.S., Hyper., E.H., Dil. Aorta, N.S.R.
Em Hi	56	F	R.F., E.H., M.S., M.I., A.I., A.F.
Gu Na	53	F	R.F., Hyper., E.H., M.S., M.I., A.F.
Mo Me	52	M	R.F., E.H., M.S., M.I., A.I., N.S.R.
Es Is	62	F	A.S., Hyper., E.H., A.F.
Ra Ma	59	F	L., A.I., An. Aorta, N.S.R.
He Fa	42	F	Hyper., E.H., Ang. Synd., N.S.R.
Id Ha	56	F	A.S., Hyper., E.H., Scler. Aorta, N.S.R.
Mo Ne	61	M	A.S., E.H., Cor. Scler., Dil. Aorta, Ang. Synd., N.S.R.
Be Ru	36	F	R.F., E.H., M.S., M.I., A.I., N.S.R.
Mo Ka	60	M	R.F., E.H., M.S., M.I., Ao.St., A.I., A.F.
Es Bu	48	F	A.S., Hyper., E.H., N.S.R.
Re Wi	56	F	Hyper., E.H., B.B.B.
Ja Ad	67	M	A.S., Hyper., E.H., Cor. Thromb., Scler. Aorta, N.S.R.
Ma Lu	60	M	A.S., Hyper., E.H., Scler. Aorta, A.F.
Is Ro	66	M	A.S., E.H., An. Aorta, B.B.B.
Ch Co	59	M	A.S., E.H., N.S.R.
Be Wi	68	M	R.F., E.H., M.S., M.I., N.S.R.

* According to "Nomenclature and Criteria for Diagnosis of Diseases of the Heart" of the New York Heart Association (1942).

A.S. (Arteriosclerosis); R.F. (Rheumatic Fever); L. (Lues); Hyper. (Hypertension); E.H. (Enlarged Heart); Cor. Scler. (Coronary Sclerosis); Cor. Thromb. (Coronary Thrombosis); Scler. Aorta (Sclerosis of the Aorta); Dil. Aorta (Dilatation of the Aorta); An. Aorta (Aneurysm of the Aorta); M.S. (Mitral Stenosis); M.I. (Mitral Insufficiency); Ao.St. (Aortic Stenosis); A.I. (Aortic Insufficiency); N.S.R. (Normal Sinus Rhythm); A.F. (Auricular Fibrillation); B.B.B. (Bundle Branch Block); Ang. Synd. (Anginal Syndrome).

ments, and the blood N.P.N. was determined at intervals of 2 weeks. The decholin sodium was used in 20 per cent solution in doses of 10 ml. or 2.0 grams. It was administered intravenously, taking 2 to 3 minutes for the injection. Most patients received 4 such doses, either alone, or in combination with mercupurin. When the two drugs were given together, they were mixed in the same syringe. The mercupurin was also injected intravenously.

RESULTS

There were, in all, 234 intravenous injections of mercupurin and 98 of decholin sodium. The results of the experiments which were satisfactorily completed have been assembled in Table II. In view of the fact that the patient was weighed

TABLE II

Comparison of decholin and mercupurin under various conditions

Drug	Dose	No. of tests	No. of patients	Wt. loss (lbs.)		
				Average	S.E.	Range
— A —						
0	0	13	10	0.4	±0.2	-1.25 to +1.0*
— B —						
Mercupurin	1 or 2 ml.	93	22	4.9	±0.39	0.5 to 15.5
Decholin	2 grams	55	22	1.5	±0.18	0 to 4.5
Mixture		38	21	6.2	±0.44	0 to 13.0
Mercupurin	1 or 2 ml.					
Decholin	2 grams					
— C —						
Mercupurin	1 ml.	31	6	5.1	±0.30	3.6 to 6.8
Mercupurin	2 ml.	31	6	7.0	±0.28	4.3 to 8.1
Mercupurin	2nd ml.	31	6	1.9†		
Decholin	2 grams	16	6	1.7	±0.36	0 to 4.5
— D —						
Mercupurin	1 ml.	57	14	4.9	±0.44	1.0 to 9.3
Decholin	2 grams	33	14	1.8	±0.23	0 to 4.5
Mixture		24	13	6.3	±0.38	3.0 to 10.3
Mercupurin	1 ml.					
Decholin	2 grams					
— E —						
Mercupurin	2 ml.	36	9	5.1	±0.66	0.5 to 15.5
Decholin	2 grams	22	8	0.9	±0.22	0 to 4.0
Mixture		14	8	5.9	±1.02	0 to 13.0
Mercupurin	2 ml.					
Decholin	2 grams					

* Control cases are only instances of gain in weight.

† Obtained by subtracting the 5.1 ml. from the 7.0 ml. S.E. (Standard Error).

and received the injection in the evening, and the loss of weight was determined the following morning, there was the possibility that in this period the effect of rest in bed, bowel movement, and food intake might result in a change in weight without drug. This was tested in 13 trials (Group A). The results show that while some gained and others lost weight, the net result was an average 0.4 pound loss of weight in the 15-hour period without drug, an amount which is close to the limit of accuracy of the scale for weighing the patients and outside of the range of the vast majority of losses of weight occurring after the diuretic agents. Since the data were not sufficient to determine the precision of this factor, the ratios in the tables were calculated on

the basis of the actual changes in weight following the use of the diuretic agents.

Mercupurin showed a failure of diuretic response in 3 per cent of 134 tests and decholin sodium failed in 19.6 per cent of 56 tests.

After the intravenous injection of 2 grams of decholin sodium, patients with cardiac edema developed a diuresis and lost an average of 1.5 pounds. This was about 30 per cent of the effect of 1 or 2 ml. of mercupurin in the same group of patients (Group B). When the decholin sodium and mercupurin were injected together, the diuresis was greater than with either alone and the increase represented essentially a summation of the two actions.

In one group of 6 patients (Group C) who were tested by both a 1 ml. and a 2 ml. dose of mercupurin, it was found that the 2 gram dose of decholin sodium produced only about 33 per cent of the effect of the first ml. of mercupurin but its effect was nearly equal to that of the second ml. of mercupurin. Its effect was, therefore, nearly equal to the extra effect of doubling the dose of mercupurin in this dosage range.

Among the patients receiving mercupurin, there were those who were more susceptible (Group D), requiring only the 1 ml. dose, and those who were more tolerant (Group E), requiring the 2 ml. dose for the production of enough diuresis to control symptoms effectively. This difference in tolerance extended also to decholin sodium since the 2 gram dose produced only one-half as much diuresis in those resistant as in those more susceptible to mercupurin. These groups also show that the combined use of decholin sodium and mercupurin produces substantially summative diuresis.

The foregoing relationships have been summarized in Table III.

The size of the liver was uninfluenced by the manner of the treatment in these cases. The average size in 14 patients (17 tests) was 9 cm. before and 9.5 cm. after the dose in the case of the mercupurin, and in 16 patients (19 tests), it was 11.4 cm. before and 11.6 cm. after the dose in the case of the decholin sodium.

The blood N.P.N. was also unchanged by the decholin sodium, average 38.4 mgm. before and 39.4 mgm. after the 4 doses in 19 cases, and none

TABLE III

Summary of results in comparison of diuretic effects of decholin and mercupurin

1. Average loss of weight in the 15-hour period (control)	0.4 lbs.
2. Average diuretic effect of 2 grams decholin sodium in percentage of that of mercupurin (1 or 2 ml.)	30 per cent
3. Average increase in diuretic effect of mercupurin (1 or 2 ml.) by simultaneous injection of 2 grams decholin sodium	26 per cent
4. Increase in diuresis produced by raising dose of mercupurin from 1 to 2 ml. in one series of patients	37 per cent
5. Diuretic effect of 2 grams decholin sodium in percentage of effect of a 1 ml. dose of mercupurin in above series of patients	33 per cent
6. Diuretic effect of 2 grams decholin sodium in percentage of effect of the mercupurin in patients requiring	
(a) 1 ml. mercupurin for about a 5 lb. diuresis (sensitive)	36 per cent
(b) 2 ml. mercupurin for about a 5 lb. diuresis (tolerant)	17 per cent

of the individual cases showed any significant changes. There were no significant changes in the urine. There were no significant changes in the blood pressure. All patients experienced the bitter taste after the injection. One patient vomited. In one case, 8 ml. of the solution of decholin sodium was injected into the perivascular tissues. It caused mild fleeting pain which subsided without inflammation or thrombosis.

SUMMARY AND CONCLUSIONS

1. The effect of decholin sodium by intravenous injection in a dose of 2 grams was compared with that of mercupurin in a group of ambulatory patients with advanced heart disease and congestive failure.

2. This dose of decholin sodium causes a moderate diuretic effect in about 80 per cent of the cases, amounting on the average to about one-third as much as that of mercupurin.

3. When the decholin is given with mercupurin, the diuresis represents the summation of the effect of the two agents.

4. Patients resistant to the diuretic action of mercupurin are likely to be resistant to that of decholin as well.

5. The 2 gram dose of decholin sodium given together with the 1 ml. of mercupurin may be

expected to produce a diuretic effect similar to doubling the dose of mercupurin.

6. The diuretic action of decholin sodium seems to be applicable to the same type of cases for which the organic mercurials are employed.

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THE RENAL CLEARANCES OF SUBSTITUTED HIPPURIC ACID DERIVATIVES AND OTHER AROMATIC ACIDS IN DOG AND MAN¹

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The efficiency with which diodrast (3, 5-di-iodo-4-pyridone-1-acetic acid) and hippuran (o-iodohippuric acid) are excreted by the renal tubules (1, 2) raises the question to what extent the organically-bound iodine in these molecules is responsible for the phenomenon of tubular excretion. Since suitable quantitative methods for the determination of unsubstituted compounds were not available, substituted derivatives of hippuric acid which could be determined by appropriate coupling reactions were prepared² and studied under conditions permitting the exact comparison of renal clearances with those of diodrast and hippuran.

Methods of comparing clearances. When two substances, both of which are excreted by the tubules, are presented to the tubular excretory mechanism simultaneously, one substance may depress the tubular excretion of the other (2, 3).³ Consequently, in the absence of information to

the contrary, the comparison of tubular clearances by the conventional simultaneous method is hazardous since, even if the two clearances are identical, it is possible that the clearance of one substance may be depressed by the presence of the other. Although essential in principle, this precaution has proved to be of minor importance with most of the compounds studied here, since "titration" experiments (unpublished), in which the plasma concentration of one compound is raised from zero in the presence of a constant concentration of the other compound, have shown that diodrast is more effective in depressing the clearance of p-hydroxyhippuric and p-aminohippuric acid than these compounds are in depressing the diodrast clearance. Consequently we have designed our experiments so that diodrast was introduced into the blood after a series of clearance determinations with the hippuric acid derivatives. Thus there are afforded three methods of calculation:

1. Comparison of successive clearances, wherein the clearance (C_X) of that compound (X) with either negligible or least depressive power is measured alone in 3 successive 10-minute periods, followed by a similar 3-period clearance (C_Y) determination of the second compound (Y), simultaneously with C_X , an interval of 20 minutes elapsing between the first and second series of clearances.

In such experiments, the plasma concentration of X has been maintained at the lowest levels compatible with analytical accuracy; for o-, m-, and p-hydroxyhippuric acids this is 2 to 4 mgm. per 100 ml., for m- and p-aminohippuric acid and p-acetylaminohippuric acid, 1 to 3 mgm. per 100 ml. Hippuran and diodrast have been used in plasma concentrations of 1 to 2 mgm. of iodine per 100 ml. With these plasma concentrations,

¹ Aided by a grant from the Commonwealth Fund. A preliminary report on this work was given before the American Physiological Society in 1941 (22).

² We are indebted to Dr. Kenneth C. Blanchard for the synthesis of o- and p-hydroxyhippuric acid, p-aminohippuric acid, 2-pyridone-1 acetic acid, p-aminophenaceturic acid, and several compounds not reported in this paper. Subsequently Merck and Company kindly prepared for us m-aminohippuric acid, p-aminomandelic acid, m-hydroxyhippuric acid, and additional p-aminohippuric acid. p-Acetaminohippuric acid and cinnamoyl glycine were prepared by Mr. John Mulvaney under Dr. Blanchard's direction. Large quantities of pyrogen-free 20 per cent solution of sodium p-aminohippurate as well as the anhydrous (reagent) free acid for the preparation of standard solutions were supplied through the courtesy of the Medical Research Division of Sharp and Dohme.

³ That hippuric acid is excreted by the tubules was indicated in preliminary experiments by the fact that the intravenous administration of sodium hippurate markedly depresses the phenol red clearance (without affecting the filtration rate) in the dog.

it is believed that the diodrast clearance is not appreciably depressed by the hippuric acid derivatives.

In this method, precaution must be taken to insure that the renal blood flow remains as constant as possible throughout the two series of observations, and that absolute errors in timing and collection of urine samples are reduced to a minimum. One disturbing physiological factor is excessive hydration to produce diuresis, which tends to cause renal hyperemia in the dog. It has been our experience that the renal plasma flow does not become constant after excessive hydration until the urine flow has fallen to below 3 ml. per minute (15 kgm. dog) and even then progressive reduction throughout 5 or 6 periods implies regression of hyperemia. At urine flows below 1.0 ml. per minute, accurate timing and complete collection of urine samples are difficult.

2. All the above errors may be avoided in some measure by comparing the successive C_X/C_{Cr_1} and C_Y/C_{Cr_2} clearance ratios (Column 8, Table IV and Column 9, Table V) in the sense $C_X/C_{Cr_1} :: C_Y/C_{Cr_2}$ (where C_{Cr_1} is the creatinine clearance in the first 3 periods and C_{Cr_2} the creatinine clearance in the second 3 periods). Alternatively, the inulin (C_{IX}) or mannitol (C_M) clearance ratio is used for this purpose in man. Here errors in timing and urine collection affect C_X and C_{Cr_1} (or C_Y and C_{Cr_2}) equally and hence cancel out.

Moreover, in the dog the filtration rate, C_{Cr} , tends to vary with the renal plasma flow, C_X (or C_Y where C_Y is approximately equal to C_X), the filtration fraction C_{Cr}/C_X changing only slightly with marked changes in C_X . Consequently, changes in the renal plasma flow tend to be eliminated when one compares the successive clearance ratios. Indeed, for the dog, we consider this to be a much more accurate method of comparison than method 1.

In man, the filtration fraction varies inversely as the renal plasma flow and the only advantage of this method, as compared with the preceding one, is the elimination of timing and collection errors.

3. Where it is known that X does not depress the clearance of Y, an adequate concentration of X may be maintained in the plasma throughout the determination of the C_Y so that there is avail-

able the simultaneous clearance ratio, C_X/C_Y , in the second series of observations. So far as errors, other than possible mutual depression of clearances are concerned, this is of course the most accurate method of the three.

Where available, all three ratios are reported in Tables IV and V.

Blanks in plasma and cells. Because of the small order of magnitude of the blanks involved in both plasma and cells, these values were determined additively: i.e., a known quantity of substance under determination was added to a suitable filtrate of plasma or cells (see Methods) and the blank determined by difference. Since it is possible that substances present in plasma filtrates may inhibit color development, in some instances at least 3 concentrations were added to aliquots of the same plasma filtrate and the resulting apparent concentrations plotted against the theoretic values. If no inhibition of color development is present, the 3 determinations should fall along a straight line with a slope of 1.0 and which either extrapolates through the zero coordinates or to a positive intercept equal to the value of a constant blank. (Such inhibition has been observed only on m-hydroxy hippuric acid as described under Methods.)

Determination of the blank was, with few exceptions, carried out in each clearance experiment on any animal or man, and an appropriate correction applied to the analytical data. A tabulation of these blank values is given in Table 1. The blank values for the p-amino coupling reaction are so small that they can be neglected with plasma concentrations of 1.5 mgm. per cent or higher of p-aminohippuric acid, etc.

Blank excretion. Twelve examinations of timed urine samples from 7 individuals indicate an excretion rate of chromogenic material behaving like p-aminohippuric acid, of 0.002 to 0.036 mgm. per minute (average 0.0083). Since at a plasma concentration of 2 mgm. per cent and with a clearance of 600 ml. per minute, $UV = 12$ mgm. per minute, this blank can be wholly neglected in clearance determinations. The identity of the compound or compounds giving the color reaction is unknown. On acid hydrolysis, the UV blank increases slightly, but remains at negligible values. The blank excretion for both "apparent" free p-aminohippuric acid and acid-hydrolyzable

TABLE I
Blanks in plasma and cells in dog and man

Compound	Dog				Man			
	Plasma		Cells		Plasma		Cells	
	No. of determinations	Range	No. of determinations	Range	No. of determinations	Range	No. of determinations	Range
		<i>mgm. per 100 ml.</i>		<i>mgm. per 100 ml.</i>		<i>mgm. per 100 ml.</i>		<i>mgm. per 100 ml.</i>
o-Hydroxyhippuric acid	2 ¹	0 to 0.28	1 ⁷	0.38	1 ¹	0 to 0.44	1 ³	0.38
o-Hydroxyhippuric acid	1 ²	0	1 ³	0.04				
m-Hydroxyhippuric acid	5 ²	0.04 to 0.06						
p-Hydroxyhippuric acid	6 ⁴	0.1 to 0.3	5 ^{3,10}	0.3 to 1.97	8 ⁴	0.13 to 0.26	6 ³	0 to 0.31
m-Aminohippuric acid	5 ²	0 to 0.06	2 ³	0 to 0.02	4 ²	0	2 ³	0 to 0.01
p-Aminohippuric acid	11 ⁵	0	6 ⁶	0 to 0.18	9 ⁵	0 to 0.10	8 ⁶	0 to 0.4
p-Aminohippuric acid	5 ²	0			5 ²	0	2 ³	0
p-Acetylaminohippuric acid	2 ⁸	0.08 to 0.11			3 ⁵	0	1 ⁶	0
p-Acetylaminohippuric acid					2 ²	0	1 ³	0
p-Aminomandelic acid	4 ²	0 to 0.08			4 ²	0 to 0.05	1 ³	0.17
p-Aminophenylsuccinic acid	1 ²	0.02	1 ³	0.02				
p-Aminophenacetic acid	3 ²	0 to 0.01						
Diodrast	9 ²	0	1 ³	0.24			3 ³	0.24 to 0.35
p-Aminobenzoic acid	2 ²	0						

¹ Precipitated with tungstate 1-5.

² Precipitated with cadmium sulfate 1-15.

³ Diluted 1-2.5 with distilled water; precipitated with cadmium sulfate 1-5.

⁴ Precipitated with cadmium sulfate 1-5.

⁵ Precipitated with trichloroacetic acid 1-10.

⁶ Diluted 1-2.5 with distilled water; precipitated with trichloroacetic acid 1-10.

⁷ Diluted 1-2.5 with distilled water; precipitated with tungstate 1-5.

⁸ Precipitated with trichloroacetic acid 1-5.

⁹ Analyzed by fusion method.

¹⁰ Direct rather than additive determination.

¹¹ This figure applies to man only and confirms Alpert (20). On dogs we have obtained so nearly 100 per cent recovery that no correction has been made for clearance comparison.

¹² White (4) gives 0.32.

conjugated material is similarly low and negligible in the dog. The blank excretion of p-phenols tends to be somewhat larger, but nevertheless can usually be neglected in both species.

Recoveries were determined in plasma by substituting 2 ml. of standard solutions of the compound under investigation for 2 ml. of water in the process of precipitating 2 ml. of plasma. A similar method was used with cells, the concentrated cells being hemolyzed before addition of the standard solution. The appropriate blank value determined on the sample of plasma or cells was deducted in calculating the recovery.

As might be expected, recovery from hemolyzed cells is less than 100 per cent, but it is nevertheless fairly uniform for any one compound. No consistent difference has been found in the behaviour of dog and human plasma and red cells

and the respective figures have been averaged in Table II.

In calculating plasma clearances, recovery has been taken at 100 per cent for all compounds except p-hydroxyhippuric acid and diodrast, where the figures 89 and 94 per cent, respectively, were used in man (for dog, see Note 11, Table II). The recovery figures shown in Table II were used in calculating the *in vivo* distribution between red cells and plasma shown in Table III.

From experience, we have found that it is important, especially when working with relatively unfamiliar compounds, to include a "blank" and a "recovery determination" in each set of experimental analyses.

In vivo distribution between red cells and plasma (Table III). It is known that although diodrast penetrates the red cell to a negligible

TABLE II
Precipitation recoveries from dog and human plasma and cells

Compound	Plasma			Cells		
	No. of determinations	Plasma concentration	Average recovery	No. of determinations	Plasma concentration	Average recovery
		mgm. per 100 ml.	per cent		mgm. per 100 ml.	per cent
o-Hydroxyhippuric acid	1 ¹	4	98			
o-Hydroxyhippuric acid	1 ²	4	100	1 ³	3	99
m-Hydroxyhippuric acid	4 ²	2	100			
p-Hydroxyhippuric acid	4 ⁴	3	89	6 ³	6 to 27	58
m-Aminohippuric acid	7 ²	2	99	4 ³	2.5	82
p-Aminohippuric acid	14 ⁵	2	101	15 ⁶	1.5 to 45	85
p-Aminohippuric acid	5 ²	2	102	2 ³	3	80
p-Acetylaminohippuric acid	5 ⁵	3	99			
p-Acetylaminohippuric acid	3 ²	2	99	1 ³	1.5	88
p-Aminomandelic acid	11 ²	2	100	6 ³	2.5	88
p-Aminophenylsuccinic acid	2 ²	2	99	1 ³	2	52
p-Aminophenaceturic acid	2 ²	2	100			
Diodrast	7 ^{2,11}	2 to 50	94	6 ³	1 to 2.5	70
p-Aminobenzoic acid	2 ²	1	99			

(For footnotes see Table I.)

extent in *in vitro* mixtures with whole blood (2), considerable penetration occurs *in vivo*, as demonstrated by White (4), who has concluded that a significant part of the diodrast excreted in the urine in the dog is extracted from the red cell during the passage of the blood through the kidney. This circumstance would of course tend to give a diodrast clearance above the true value of the renal plasma flow.

We have likewise found that although p-aminohippuric acid does not penetrate the red cell to

a significant extent in *in vitro* mixtures of dog or human blood, there is some penetration in dog blood *in vivo*. This is true to a greater or less extent of all the compounds listed in Table III, though strangely the ultimate distribution, under conditions where the plasma concentration has been fairly constant for 20 to 90 minutes, is never one of equality per unit of water. This failure to reach an equal distribution per unit of water was also noted by White in the case of diodrast.

TABLE III
In vivo distribution between red cells and plasma

Compound	Dog				Man			
	Method	No. of determinations	Plasma concentration	Cell Plasma distribution	Method	No. of determinations	Plasma concentration	Cell Plasma distribution
			mgm. per 100 ml.	per 100 grams water			mgm. per 100 ml.	per 100 grams water
o-Hydroxyhippuric acid	Direct ¹	17	3 to 10	0.3	Direct ¹	2	1.5	0.3
p-Hydroxyhippuric acid	Additive ¹	6	0.9 to 2.7	0.41	Additive ¹	8	2.5 to 5.2	0.16
m-Aminohippuric acid	Direct ^{2,4}	13	1.5 to 15	0.5	Additive ²	16	2.5	0
p-Aminohippuric acid					Additive ²	16	1.5	0
p-Acetylaminohippuric acid					Additive ²	4	3	0
p-Acetylaminohippuric acid	Additive ²	5	1.3 to 18	0.16	Additive ²	5	1.5	0
p-Aminomandelic acid	Additive ²	1	3.5	0.19	Additive ²	5	1.5	0.16
p-Aminophenylsuccinic acid	Direct ³	8	1 to 2	0.73	Additive ³	10	1.5	0.35 ¹¹
Diodrast	Direct ³	12	1 to 32	0.65	Direct ³	6	1.5 to 3.5	0.49
Hippuran	Direct ³	6	1 to 4	0.66				
Iopax								

(For footnotes see Table I.)

The tendency of all compounds to penetrate the red cell is much less in man than in the dog, and indeed no definite penetration was ever demonstrable in man in the case of m-aminohippuric acid, p-aminohippuric acid, or p-acetylaminohippuric acid. The data on p-aminohippuric acid in Table V include 2 prolonged experiments (11 and 12 periods, respectively) with pyrogenic renal hyperemia (5), a fairly constant concentration of the acid being present in the blood for 3 to 4 hours. Despite this prolonged exposure, no detectable quantity of p-aminohippuric acid was present in the cells. These experiments demonstrate that the increase in diodrast and p-aminohippuric acid clearances during pyrogenic hyperemia is referable solely to renal hyperemia and not in any measure to increased extraction of the compound from the red cell. Indeed, the general identity of these clearances indicates that no significant quantity of diodrast moves out of the red cell during the renal passage of the blood.

Comparison of clearances in the dog. The pertinent data on the renal clearances of the substances studied here are given in Table IV.

Essentially identical clearances are shown by m-hydroxyhippuric acid, p-hydroxyhippuric acid, m-aminohippuric acid, p-aminohippuric acid, p-acetylaminohippuric acid, 2-pyridone-1-acetic acid, cinnamoylglycine, hippuran, and diodrast.

This conclusion would be reached whether one considered the ratios of successive clearances (Column 7), the ratio of the successive filtration fractions (Column 8), or the ratio of the simultaneous clearances (Column 9), except in the case of cinnamoylglycine the clearance of which appears to be substantially depressed by the presence in the plasma of p-aminohippuric acid.

It is thus clear that the presence of iodine in hippuran (o-iodohippuric acid) is not essential to the process of tubular excretion, but that the necessary properties are possessed by the parent hippuric acid molecule. Similarly, 2-pyridone-1-acetic acid (of which a sufficient quantity for only 2 experiments was available) appears to be excreted as efficiently as is the iodo derivative, iopax (5-iodo-2-pyridone-1-acetic acid) (6). The identity of the above clearances conforms with the premise (7, 2) that the limiting circumstance in renal excretion of diodrast (or of any one of the above substances) at low plasma concentrations

is the effective blood flow to the renal tubules (which supposedly closely approximates the total renal blood flow⁴) rather than a limitation in the tubular excretory mechanism.

Clearances significantly lower in the dog than those of diodrast or the above hippuric acid derivatives are shown by o-hydroxyhippuric acid, p-aminophenacetic acid, iopax, and p-aminobenzoic acid. No interpretation suggests itself to account for the difference in behavior between o-hydroxyhippuric acid and the p- and m- derivatives or for the behavior of iopax, though presumably it represents some limitation in the tubular excretory mechanism. p-Aminobenzoic acid, when infused at a constant rate, shows a tendency for the clearance to rise with time, and in 1 experiment where a single injection of p-aminobenzoic acid was given intravenously and the clearance was determined in successive periods on a falling plasma concentration, the clearance rose substantially above that of creatinine. We interpret the rising clearance as reflecting the formation in the body of p-aminohippuric acid from p-aminobenzoic acid. Similarly, p-aminophenylsuccinic acid shows a rising clearance starting in excess of the creatinine clearance, implying metabolism to an unidentified compound having a high clearance value.

p-Aminomandelic acid has a clearance very close to that of creatinine. Whether the discrepancy between the two clearances is attributable to systematic error in the determination of one or the other substances or indicates tubular excretion of the acid cannot be said from the available data. Unfortunately, necessary cessation of work prevented further examination.

Comparison of clearances in man (Table V)⁵. The same methods of comparison as are described

⁴ White (4) reports the average diodrast renal extraction ratio in the dog as 0.74, while Corcoran, Smith and Page (23) obtained 0.87. More recently Bradley and his coworkers (personal communication) and Stead and his coworkers (personal communication) have independently observed the renal extraction ratio of diodrast in man to average 0.87. Phillips *et al.* (24) report the extraction ratio of p-aminohippuric acid in the dog to be 0.85.

⁵ We are indebted to Dr. William Goldring, Dr. Herbert Chasis, Dr. Hilmert Ranges, and Dr. Stanley Bradley for assistance in the observations on man reported in Table V, and to Miss Martha Barrett and Miss Claire Lawler for the inulin and mannitol determinations.

TABLE IV

Comparison of successive and simultaneous clearance ratios in the dog

Each experiment consists of 2 series of 3 clearance periods each; in the first series, the clearance of substance X was observed alone, relative to the creatinine clearance, after which substance Y was introduced (second series). Thus there are available the ratio of the successive clearances of X and Y (column 7), the ratio of the successive filtration fractions (column 8), and the ratio of the simultaneous clearances (column 9). All clearances have been corrected to 1 square meter body surface area.

1 Dog no.	2 Date	3 C_X	4 C_Y	5 $\frac{CCR_1}{C_X}$	6 $\frac{CCR_2}{C_Y}$	7 Successive clearance ratio $\frac{C_X}{C_Y}$	8 $\frac{CCR_2/C_Y}{CCR_1/C_X}$	9 Simultaneous clearance ratio $\frac{C_X}{C_Y}$
o-Hydroxyhippuric acid (X) and diodrast (Y)								
3	12- 2-40	173	208	0.37	0.26	0.83	0.72	0.65
1	12- 4-40	84	133	0.48	0.31	0.63	0.64	0.41
3	12-17-41	110	175	0.52	0.31	0.71	0.64	0.56
Average						0.72	0.66	0.53
m-Hydroxyhippuric acid (X) and diodrast (Y)								
4	11-26-41	154	150	0.26	0.28	0.92	1.05	0.97
1	11-28-41	150	129	0.20	0.24	1.10	1.22	1.16
3	12- 1-41	180	170	0.29	0.33	1.11	1.10	1.14
4	12-15-41	163	152	0.29	0.30	1.10	1.04	1.06
Average						1.03	1.10	1.08
p-Hydroxyhippuric acid (X) and diodrast (Y)								
4	10- 7-40	196	202	0.25	0.25	0.92	0.98	
3	10-16-40	246	258	0.30	0.29	0.95	0.98	
3	11-13-40	235	230	0.29	0.28	1.02	0.98	
5	5-24-40	150	152	0.37	0.37			0.99
Average						0.98	0.98	0.99
p-Hydroxyhippuric acid (X) and hippuran (Y)								
2	12- 9-40	170	160	0.29	0.28	1.08	0.98	0.92
3	1- 7-41	170	178	0.31	0.31	0.99	1.01	0.83
4	4-24-41	159	128	0.31	0.37	(1.24) ²	(1.18) ²	1.01
Average						1.03	1.00	0.92
p-Hydroxyhippuric acid (X) and p-acetylaminohippuric acid (Y)								
4	12-11-40	174	139	0.31	0.35	(1.25) ¹	(1.12) ¹	0.96
m-Aminohippuric acid (X) and diodrast (Y)								
3	10-24-41	213	271	0.25	0.23	(0.79) ¹	(0.90) ¹	1.01
2	10-27-41	149	143	0.26	0.25	1.05	0.94	0.97
3	11- 3-41	146	173	0.28	0.28	(0.84) ¹	1.00	1.00
2	12- 3-41	143	148	0.29	0.31	0.90	1.07	1.07
Average						0.98	1.00	1.01
p-Aminohippuric acid (X) and diodrast (Y)								
1	1- 9-41	135	146	0.28	0.26	0.93	0.93	0.83
2	1-13-41	169	174	0.28	0.25	0.97	0.92	0.93
3	1-16-41	197	207	0.33	0.32	0.95	0.96	0.94
4	5-12-41	154	149	0.32	0.31	1.03	0.96	0.99
4	6-12-41	140	122	0.30	0.33	1.15	1.10	1.04
2	10- 1-41	179	162	0.26	0.27	1.11	1.01	1.07
Average						1.02	0.95	0.97

TABLE IV—Continued

1	2	3	4	5	6	7	8	9
Dog no.	Date	C _X	C _Y	$\frac{CCR_1}{C_X}$	$\frac{CCR_2}{C_Y}$	Successive clearance ratio C _X /C _Y	$\frac{CCR_2/C_Y}{CCR_1/C_X}$	Simultaneous clearance ratio C _X /C _Y
p-Acetylaminohippuric acid (X) and diodrast (Y)								
3	12-16-40	193	256	0.29	0.23	(0.75) ¹	(0.79) ¹	0.94
1	12-19-40	129	130	0.27	0.29	0.99	1.10	
2	1-2-41	168	150	0.24	0.27	1.12	1.06	0.96
4	12-22-41	163	155	0.25	0.26	0.97	1.01	0.94
3	12-23-41	252	256	0.29	0.30	0.96	1.01	1.02
Average						1.01	1.04	0.97
p-Aminophenaceturic acid (X) and diodrast (Y)								
1	22-6-42	104		0.31				
4	2-10-42	145	168	0.32	0.28	0.86	0.88	0.78
2	2-12-42	180	187	0.28	0.22	0.96	0.79	0.68
4	4-17-42	139	187	0.39	0.36	0.74	0.79	0.67
Average						0.86	0.82	0.71
Iopax (X) and p-hydroxyhippuric acid (Y)								
4	1-20-41	124	148	0.41	0.36	0.84	0.87	0.97
1	1-22-41	54	116	0.61	0.33	0.47	0.54	0.69
4	4-28-41	166	261	0.38	0.27	0.64	0.70	0.81
Average						0.65	0.70	0.82
2-Pyridone-1-acetic acid (X) and p-aminohippuric acid (Y)								
3	5-7-42	162	185	0.34	0.39	0.88	1.12	0.83
1	5-12-42	173	143	0.21	0.25	1.21	1.17	1.13
Average						1.04	1.15	0.98
Cinnamoylglycine (X) and p-aminohippuric acid (Y)								
1	3-6-42	106	127	0.27	0.24	0.83	0.92	0.70
3	4-27-42	153	139	0.26	0.28	1.10	0.90	0.70
Average						0.97	0.91	0.70
p-Aminophenylsuccinic acid (X) and creatinine (Y)								
2	11-5-41	Simultaneous values of C _X /C _Y rose from 1.05 to 1.47						
4	11-7-41	Simultaneous values of C _X /C _Y rose from 1.61 to 1.86						
p-Aminobenzoic acid (X) and creatinine (Y)								
1	11-10-41	25	42	0.59				
2	11-13-41	Simultaneous values of C _X /C _Y rose from 1.10 to 1.70						
p-Aminomandelic acid (X) and creatinine (Y)								
1	10-8-41	38	33	1.15				
4	10-16-41	54	52	1.04				
1	10-22-41	37	36	1.03				
Average				1.07				

¹ Clearance of X increased in second series of test, indicating increased renal plasma flow and invalidating comparison of successive clearances or successive clearance ratios.

² Clearance of X increased so markedly in second series of test that discrepancy is clearly attributed to receding hyperemia.

TABLE V

Comparison of successive and simultaneous clearance ratios in man

Each experiment consists of 2 series of clearance periods; in the first series, the clearance of substance X was observed alone, after which substance Y was introduced (second series). Thus there are available, relative to the inulin (or mannitol) clearance, the ratio of the successive clearances (column 9), the ratio of the successive filtration fractions (column 10), and the ratio of the simultaneous clearances (column 11). All clearances have been corrected to 1.73 square meter surface area.

1	2	3	4	5	6	7	8	9	10	11
Subject	Date	Number of determinations	C _X	C _Y	$\frac{C_{IN_1}}{C_X}$	$\frac{C_{IN_2}}{C_Y}$	Successive clearance ratio $\frac{C_X}{C_Y}$	$\frac{C_{IN_2}/C_Y}{C_{IN_1}/C_X}$	Simultaneous clearance ratio $\frac{C_X}{C_Y}$	Diagnosis
o-Hydroxyhippuric acid (X) and diodrast (Y)										
I.N.	12-13-40	8	269	428	0.35	0.21	0.63	0.68	0.61	Subdeltoid bursitis
J.K.	4- 4-41	6	487	686	0.23	0.16	0.70	0.69		Pneumonia
						Average 0.66		0.68		
p-Hydroxyhippuric acid (X) and diodrast (Y)										
S.W.	11-21-40	10	364	375	0.20	0.21	0.97	1.02	1.01	Hypertension
W.O.	3-31-41	6	532	845	0.24	0.15	(0.63) ¹	(0.63) ¹		Alcoholism
C.A.	4- 9-41	6	702	692	0.18	0.19	1.02	1.06		Syphilis
M.J.	4-18-41	6	478	472	0.23	0.23	1.01	0.99		Hypertension
B.D.	4-21-41	6	544	574	0.17	0.17				Multiple sclerosis
M.J.	4-23-41	6	487	432	0.22	0.24	1.13	1.11		Hypertension
V.B.	1- 2-41	8	Pyrogenic hyperemia			Average 1.03		1.05	0.98	Grippe
								1.00		
p-Hydroxyhippuric acid (X) and hippuran (Y)										
P.R.	4-28-41	6	554	586	0.18	0.15	0.94	0.87		Gastric ulcer
H.B.	4-30-41	6	722	670	0.18	0.17	1.08	0.99		Arthritis
A.R.	5- 9-41	6	395	391	0.23	0.22	1.01	0.92		Rheumatic fever
m-Aminohippuric acid (X) and diodrast (Y)										
K.F.*	11- 7-41	6	686		0.17					Essential hypertension
L.R.*	11-10-41	6	474	462	0.19	0.21	1.03	1.11	0.99	Cholelithiasis
A.C.*	12- 5-41	4	406	481	0.17	0.14	0.84	0.81	0.96	Nephritis
L.A.*	11-14-41	6	592	528	0.18	0.18	1.12	1.01	1.02	Bronchitis
						Average 0.99		0.97	0.99	
p-Aminohippuric acid (X) and diodrast (Y)										
H.B.	4- 2-41	5	695	649	0.23	0.18	1.07	0.77		Arthritis
J.L.	4-14-41	6	509	591	0.24	0.20	0.86	0.81		Grippe
J.M.	4-16-41	6	442	534	0.22	0.20	0.83	0.92		Lumbar sacral sprain
R.C.	5-13-41	6	598	609	0.14	0.14	0.98	0.60		G.I. pathology
J.B.	5-14-41	6	588	603	0.20	0.18	0.98	0.89		Glomerular nephritis
C.B.	5-16-41	6	491	511	0.23	0.21	0.96	0.91		Essential hypertension
P.H.	10-13-41†	12	Hyperemia						0.98	Chronic duodenitis
C.B.	10-20-41†	11	Hyperemia						1.01	Essential hypertension
S.L.*	7-16-42	6	574	604	0.18	0.18			1.02	Essential hypertension
F.T.*	1- 6-43†	7	188	138	0.23	0.31			0.99	Duodenal ulcer
C.B.I.*	1-22-43†	7	334	309	0.21	0.22			1.32	Glomerular nephritis
P.L.*	1-27-43†	7	424	452	0.20	0.19			1.08	Essential hypertension
M.A.*	2-12-43	2	30	26	0.67	0.70	1.15	1.04	0.94	Essential hypertension
								1.06		Chronic diffuse glomerular nephritis
J.V.*	2-15-43	6	678	512	0.19	0.22	1.32	1.15	1.10	Chronic diffuse glomerular nephritis
H.L.*	1-15-43†	7	39	51	0.28	0.22			0.78	Terminal chronic diffuse glomerular nephritis
F.T.*	1-18-43†	6	178	158	0.19	0.22			1.12	Chronic diffuse glomerular nephritis

TABLE V—Continued

1	2	3	4	5	6	7	8	9	10	11
Subject	Date	Number of determinations	C _X	C _Y	$\frac{C_{IN_1}}{C_X}$	$\frac{C_{IN_2}}{C_Y}$	Successive clearance ratio $\frac{C_X}{C_Y}$	$\frac{C_{IN_2}/C_Y}{C_{IN_1}/C_X}$	Simultaneous clearance ratio $\frac{C_X}{C_Y}$	Diagnosis
p-Aminohippuric acid (X) and diodrast (Y)—Continued										
A.P.*	2-19-43	4	415	437	0.20	0.20	0.95	1.00	1.02	Essential hypertension
A.P.*	2-22-43	6	473	536	0.19	0.17	0.88	0.91	0.94	Essential hypertension
C.S.*	2-24-43	6	11	10	0.40	0.82	1.07	(2.04) ¹	1.06	Chronic diffuse glomerular nephritis
J.O.*	2-26-43	6	410	390	0.22	0.24	1.05	1.08	1.00	Cardiorenal disease
J.O.*	3- 1-43	6	393	407	0.23	0.20	0.97	0.89	0.88	Cardiorenal disease
M.S.*	3- 5-43	6	504	484	0.18	0.21	1.04	1.14	0.97	Cardiac hypertension
M.S.*	3- 8-43	6	447	340	0.19	0.24	1.32	1.30	1.21	Acute diffuse glomerular nephritis
A.S.*	5- 5-43	9	850	922	0.13	0.12	0.92	0.89	0.88	Duodenal ulcer
A.S.*	5-10-43	12	954	847	0.12	0.12	1.13	0.97	0.93	Duodenal ulcer
A.S.*	5-14-43	6	920	962	0.11	0.10	0.96	0.95	0.93	Duodenal ulcer
P.Mc.*	5-17-43†	6	370	391	0.19	0.18			0.95	Meningitis
P.C.*	5-19-43†	10	442	470	0.16	0.15			0.94	Essential hypertension
P.C.*	5-21-43†	3	446	422	0.18	0.19			1.06	Essential hypertension
B.V.*	5-24-43†	3	787	684	0.14	0.16			1.14	Lower back pain
M.R.*	6- 2-43*	3	784	605	0.13	0.16			1.30	Rheumatoid arthritis
M.R.*	6- 4-43†	3	762	675	0.13	0.15			1.13	Rheumatoid arthritis
W.D.*	6- 7-43†	3	453	422	0.18	0.20			1.07	Sacro-iliac sprain
A.W.*	6- 9-43†	3	368	370	0.19	0.19			1.00	Essential hypertension
Average							1.02	0.95	1.03	
p-Acetylaminohippuric acid (X) and diodrast (Y)										
M.D.	4- 7-41	6	692	664	0.21	0.21	1.03	1.00		Lung abscess
S.A.*	1- 7-42	6	603	707	0.20	0.16	(0.83)	(0.79)	(0.56) ¹	Deviated nasal septum
E.B.*	2- 4-42	6	554	516	0.16	0.17	1.25	1.04	1.22	Hypertension
E.B.*	2- 6-42	6	503	497	0.17	0.17	1.01	1.01	0.91	Hypertension
A.S.*	2-11-42	6	Reflex ischemia						0.99	Peptic ulcer
Average							1.10	1.02	1.04	
p-Aminomandelic acid (X) and mannitol (Y)										
B.J.	10-10-41	7	78	89					0.88	P.I.D.
L.Mc.	11- 3-41	8	95	99					0.96	Grippe
A.C.	11-17-41	6	54	57					0.94	Nephritis
I.N.	11-18-41	11	81	83					0.97	Subdeltoid bursitis
M.N.	2-13-42	11	102	105					0.97	Hypertension
A.M.	2-16-42	15	62	71					0.88	Essential hypertension
E.F.	2-20-42	13	69	70					0.99	Essential hypertension
Average								0.94		

¹ Aberrant result possibly attributable in the case of W.O. and S.A. to marked reduction in blood flow, and in the case of C.S. to marked renal hyperemia, in the second series of successive clearances.

* Mannitol clearance used instead of inulin.

† Diodrast and PAH done simultaneously, no successive clearance.

above for the dog, were used in comparing clearances in man. The subjects were convalescent patients on the Third (New York University) Medical Division of Bellevue Hospital. Appropriate priming and sustaining infusions were given as sterile saline solutions of the sodium salts. These solutions were sterilized by ultra-filtration through a Jena bacteriological sintered

glass filter, or by heating to 95° C. for a short period.

p-Hydroxyhippuric acid, m-aminohippuric acid, p-aminohippuric acid, p-acetylaminohippuric acid, diodrast, and hippuran show essentially identical clearances by any of the three methods of comparison. The identity of the p-aminohippuric acid and the diodrast clearances is maintained in

a variety of diseased states, even when the clearance is reduced by hypertensive disease or chronic glomerular nephritis.

o-Hydroxyhippuric acid shows a clearance significantly lower than that of diodrast, but one which by its magnitude relative to the inulin clearance indicates substantial tubular excretion.

The clearance of p-aminomandelic acid is close to the filtration rate. Although systematic errors of a slight magnitude cannot be ruled out, the deviations between this clearance and that of inulin, especially in long experiments, indicate that the process of excretion of this compound is complex.

It can be said in brief that the human and the dog kidney respond alike to the compounds studied here, as is the case with all compounds so far examined quantitatively except creatinine (3, 25).

*Determination of pK'_a .*⁶ As a matter of possible interest in relation to tubular excretion, the dissociation constant of the acidic group of a number of these compounds was determined by titration with NaOH and measurement of $(-\log a_{H^+})$ by the glass electrode (see Table VI). In the calculation, $\log(a_{H^+})$ is taken as equal to $\log(H^+) + \log\gamma_{H^+}$, where $-\log\gamma_{H^+}$ in dilute solution is approximately 0.05. Total anion (A^-) is taken as $(H^+) + (B^+)$, where (B^+) is the equivalent concentration of base added. The concentration of undissociated acid (HA) is taken as (total acid) $-(A^-)$. With these substitutions,

$$pK'_a = pH + \log \frac{(A^-)}{(HA)}$$

The pK'_a values are given in Table VI. In the case of p-aminophenylsuccinic acid, the neutralization curve is nearly linear beyond $(B^+)/ (HA) = 0.5$, indicating that the two pK' 's do not differ greatly in magnitude, and computation of these values was not attempted.

pK'_{a_2} was determined in the case of o-hydroxyhippuric and m-hydroxyhippuric acids to be 8.40 and 9.26, respectively. The effect of 0.16 M NaCl was examined in the case of hippuran,

⁶ We are indebted to Dr. Barbara A. Parker for the determination of the pK' 's of hippuran, diodrast, and iopax in water and in 0.16 M NaCl, and to Mr. Walter A. Bastedo of the Research Laboratory, Merck and Company, for all other determinations of this value.

TABLE VI

Dissociation constants of substituted hippuric acids, etc.
(Determinations made at 23° to 25° C.)

Acid	Initial total acid M $\times 10^3$	pK'_a
Diodrast	3.43	2.71
Clearances equal to diodrast:		
m-Hydroxyhippuric acid	32.10	3.58
m-Aminohippuric acid	29.40	4.18
p-Aminohippuric acid	19.43	3.83
p-Acetylaminohippuric acid	14.70	3.70
2-Pyridone-1-acetic acid	26.00	2.94
Cinnamoylglycine	4.60	3.57
Hippuran	10.06	3.63
Clearances less than diodrast:		
o-Hydroxyhippuric acid	21.70	3.56
Iopax	7.12	2.99
p-Aminophenaceturic acid	26.00	ca. 4.66
p-Aminomandelic acid	9.12	4.62
Clearance not determined:		
p-Iodomandelic acid	13.40	3.17

iopax, and diodrast, and showed a reduction in pK'_a of -0.22 , -0.04 , and -0.17 units, respectively. (In the NaCl solutions, $-\log\gamma_{H^+}$ is taken as 0.10.)

There is no correlation between pK'_a and the manner in which these substances are handled by the kidney. Recognizing that phenol red ($pK' = 7.9$) is also abundantly excreted by the renal tubules in man (8), it appears that within wide limits the acidic strength has little bearing on whether a substance will or will not be handled in this manner.

Determination of filterable p-aminohippurate in plasma. Attempts were made to measure the ultrafilterable p-aminohippurate in dog and human plasma by the collodion bag method previously used in this laboratory in connection with studies of phenol red, diodrast, etc. (9), but we were unable to obtain reproducible results. (Shannon has adduced evidence that constituents of parlodion react with aryl amines, possibly by way of nitration, with the consequence that dialysis experiments with such compounds in parlodion bags give erratic results. Personal com.) We then decided to obtain the necessary data directly in man by following the rate of excretion on a falling plasma curve, with the plasma concentration always above the level required to effect saturation of the tubules. It will be seen from the following equation

$$T_{MPAH} = U_{PAHV} - C_{IN}P_{PAHb}$$

that if U_{PAHV} is plotted against $C_{IN}P_{PAH}$, the points should fall in a straight line (assuming b

is constant), the slope of which will be equal to b , the percentage of PAH in the plasma which is ultrafilterable (here b corresponds to FW in the equation of Smith, Goldring, and Chasis (2)), and that the intercept on the $U_{PAH}V$ axis will be equal to Tm_{PAH} . In the above calculations, the best straight line was fitted by the method of least squares to the data obtained from 8 successive clearance periods, with the plasma concentration falling roughly in the range from 100 down to 25 mgm. per cent. The average value thus determined in 11 subjects was 0.83. This value is close enough to unity so that changes in b , related to changes in P_{PAH} in the range of 25 and 100 mgm. per cent, can be neglected in the calculations of Tm_{PAH} , i.e., the average value of 0.83 can be used with little error in the calculation, as in the case of diodrast. Unfortunately, we were unable to examine the effect of plasma protein concentration on b , but for practical purposes or except in special studies we believe that neglect of this factor will result in a negligible error in Tm calculation.

Conjugation of the p-amino group. The question of possible conjugation on the p-amino group is important since at least one conjugate, p-acetylaminohippuric acid, is also excreted by the tubules; conjugation would occlude the p-amino position during analysis for p-aminohippuric acid and any unanalyzed conjugate derivative present in the plasma during the determination of p-aminohippuric acid Tm might contribute to saturation of the tubules and thus reduce the Tm value of the latter. Conjugation would probably be of no importance with respect to p-aminohippuric acid clearance determination since there is no reason to believe that the conjugate derivative would interfere with the excretion of p-aminohippuric at low plasma levels, while it would not appear in the analytical method for the latter compound as applied to either plasma or urine.

Twenty-six samples of plasma and 27 samples of urine, collected during routine determination of p-aminohippuric acid Tm in man (10), analyzed by the volumetric flask method (see Methods), showed no significant amount of conjugated derivative, the average ratio of free to total acid being 1.00 for both the blood and urine series. (The plasma concentration ranged from 31 to 89 mgm. per cent, in some cases increasing, in others

decreasing, at moderate rates during the Tm determination.) It is therefore concluded that during the infusion of p-aminohippuric acid in the concentrations used for Tm determination, the absolute quantity of material conjugated in the p-position is so small as to be negligible, relative to the total quantity undergoing excretion.

However, when a single dose of 1.0 gram of sodium p-aminohippurate was given orally to a normal subject and the total urine collected for 6 hours, 65 per cent of the administered material was conjugated. In 2 subjects during a p-aminohippuric acid clearance determination, the extent of conjugation in 3 urine samples each was 9.9, 12.6, 12.8, and 7.6, 9.1, 11.5 per cent respectively. It is clear that when the body is not presented with an overwhelming quantity, the proportion conjugated is significant. This observation is in line with the known conjugation of p-aminobenzoic acid (11, 12) and of sulfanilamide (11, 13, 14) in man.

Examination of dog urine by open tube hydrolysis and corrected for blank excretion during a preliminary period after the intravenous administration of 200 mgm., showed no appreciable conjugation. It thus appears that conjugation of p-aminohippuric acid follows the behavior of p-aminobenzoic acid (11) and of sulfanilamide (11, 14), in that conjugation of the p-amino group does not occur in the dog.

Adverse physiological reactions. Five-tenths of a gram per kgm. of sodium p-aminohippurate can be given to dogs intravenously by syringe over a period of 5 to 10 minutes with no adverse reaction. Repeated determinations of clearances and Tm values have revealed no disturbance of renal function in dogs examined over several months. Administration of large quantities to man for Tm determination is described elsewhere (10).

Use of p-aminohippuric acid for the evaluation of effective renal blood flow and tubular excretory mass. In view of the following facts, sodium p-aminohippurate (PAH) appears to be suitable for the evaluation of those aspects of renal function which have hitherto been evaluated by the use of diodrast:

(a) At low plasma levels, the clearance is identical with that of diodrast, and hence equally valid as a measure of the effective renal plasma flow.

(b) The chemical determination is extremely simple, using reagents available in all clinical laboratories, the method as here described giving quantitative and easily reproducible values from suitable plasma filtrates and urine dilutions.

(c) The endogenous plasma and urine blanks are negligibly small.

(d) It does not penetrate the human red cell *in vivo*, even on prolonged infusion, and hence any possible error attributable to extraction from the blood cell during passage through the kidneys is obviated.

(e) It is non-toxic, and can be used for the evaluation of the tubular excretory mass by the saturation method.

(f) It is less extensively bound to plasma proteins than is diodrast (FW = 0.83 as compared with 0.73 for the latter), and hence errors involved in the estimation of the percentage of free and filterable material in the plasma are of less practical significance.

A comparison of the maximal rate of tubular excretion of p-aminohippuric acid (Tm_{PAH}) with the maximal rate of tubular excretion of diodrast (Tm_D) in the normal and diseased human kidney will be presented in a following paper.⁷

SUMMARY

Methods are described for the determination of hydroxy- and amino-substituted hippuric acid derivatives in urine and in protein-free filtrates of plasma. The chromogenic blanks, as given by these methods, have been determined in protein-free filtrates from plasma and red cells in dog and man, and recoveries of the compounds from plasma and red cells have been determined in both species. The *in vivo* distribution between red cells and plasma of most of the compounds is reported for both species. It is noteworthy that

⁷ p-Aminohippuric acid Tm has been measured in 3 dogs (FW = 0.87 to 0.92), the ratio of this value over diodrast Tm determined within an interval of several weeks and calculated on a molar basis being 1.00, 1.10, and 0.93. Less reliable data indicate that the molar ratio of p-hydroxyhippuric acid Tm (FW = 0.77 to 0.815) to diodrast Tm in 3 dogs was 1.18, 0.59, and 0.84. From comparative data on man (10), it is clear that a fixed molar value of Tm for different compounds is not to be anticipated in different species, although the molar ratio in one species appears to be fairly constant.

the human red cell is much less permeable *in vivo* to all the compounds studied than is the dog red cell, and that in no case, even after prolonged infusion, does the cell/plasma distribution ratio (per unit of water) approach unity.

The plasma renal clearances have been compared in the dog by (a) the ratio of the successive clearances in two closely following sets of clearance determinations, each comprised of three clearance periods; (b) the ratio of successive filtration fractions relative to creatinine, inulin, or mannitol, in these two sets of successive clearance periods; and (c) the ratio of the simultaneous clearances.

The clearances of (1) m-hydroxyhippuric acid, (2) p-hydroxyhippuric acid, (3) m-aminohippuric acid, (4) p-aminohippuric acid, (5) p-acetylaminohippuric acid, (6) 2-pyridone-1-acetic acid, (7) cinnamoylglycine, (8) diodrast (3-5 di-iodo-4-pyridone-1-acetic acid), and (9) hippuran (o-iodohippuric acid) are essentially identical in the dog. Only compounds 2, 3, 4, 5, and 9 have been examined in man, and here too the clearances are identical. This fact conforms with the premise that the limiting circumstance in the tubular excretion of diodrast (or of any one of these substances) is the available blood flow to the renal tubules (approximating the total renal blood flow), rather than a limitation in the tubular excretory mechanism.

The fact that the clearances of p-aminohippuric acid, which does not penetrate the red cell *in vivo* in man, and of diodrast which does penetrate, are identical under all conditions, including renal hyperemia, indicates that no significant quantity of diodrast moves out of the red cell during the renal passage of the blood.

The clearances of o-hydroxyhippuric acid, p-aminophenylaceturic acid, iopax, and cinnamoylglycine in the dog, although greater than the creatinine clearance, are significantly less than the clearances of diodrast and substituted hippuric acids. The o-hydroxyhippuric acid clearance is similarly lower than the diodrast clearance in man. This deficiency in clearance is presumably related to limitations in the tubular excretory mechanism.

The p-aminobenzoic acid clearance in the dog is initially less than that of creatinine, indicating tubular reabsorption (plasma binding has not been studied) but on prolonged infusion, the clearance

contained in the second matched colorimeter tube, and this 100 setting (or the corresponding air reading if the blank is stable) checked frequently during a series of determinations. The zero setting of the galvanometer should be checked daily.

In no case should a plasma blank be used for the 100 setting of the galvanometer. If a plasma blank is present, it should be determined additively, as described in the text, and applied to the recovery determination as well as to the unknowns. Otherwise large errors may be introduced by a deficient recovery in plasma precipitation obscured by a compensating plasma blank.

o- and *m*-Hydroxyhippuric acids. The following method is a modification of that of Gibbs (16):

(a) Veronal buffer: a 2 per cent sodium veronal solution adjusted to pH 9.1 with HCl and NaOH.

(b) 2,6-Dichloroquinonechloroimide solution, 25 mgm. per cent in absolute alcohol. This solution should be freshly prepared and centrifuged if not clear. It will keep for only a few hours.

To 10 ml. of plasma filtrate or diluted urine, add 2 ml. of (a) and 2 ml. of (b), shaking the mixture after the addition of each reagent. Solution (b) should be added immediately after (a), since hydroxy acids deteriorate rapidly in alkaline solution. A blank of 10 ml. of water is treated in the same manner and used for the 100 setting of the colorimeter. Maximal absorption of the indophenol is at 600 mu. but a 635 mu. Rubicon filter is satisfactory. A series of standards prepared from 10 ml. samples of 0.05 to 0.30 mgm. per cent solutions of *o*-hydroxyhippuric acid, or 0.1 to 0.6 mgm. per cent solutions of *m*-hydroxyhippuric acid should be prepared each time, using the same alcoholic chloroimide solution as is used in the unknowns. Maximal color development is attained in 30 minutes with *o*-hydroxyhippuric acid and in 60 minutes with *m*-hydroxyhippuric acid.

When increasing amounts of *m*-hydroxyhippuric acid are added to aliquots of the same plasma filtrate, and the resulting apparent concentrations are plotted against the concentrations in equivalent aqueous dilutions, the slope of the curve is 0.82, indicating that there is present in the filtrate something that uniformly inhibits color development. This inhibition has been taken in account in the calculation of both plasma recoveries and clearances of this compound. In addition, there is a small, positive intercept, indicating a true blank.

p-Hydroxyhippuric acid. This method is based on that of Arnow (17), and gives somewhat greater color than does Arnow's procedure.

(a) 20 per cent HgSO_4 in 5N H_2SO_4

(b) 0.2 per cent NaNO_2 solution

To 5 ml. of plasma filtrate or diluted urine in a 25 by 200 mm. heavy-walled Pyrex tube, add 7 ml. of a fresh mixture containing 3 parts of (a) and 4 parts of (b). Close the tube with a glass tear and immerse in boiling water for 4½ minutes. Cool in water at room temperature, shake and read on the colorimeter with a 490 mu. filter, using a blank prepared from 5 ml. of water and 7 ml. of reagent and treated as above for the 100 setting.

The color is stable for at least 1 hour. Although the standard curve is fairly reproducible, it is desirable because of the short heating period to include standards (5 ml. each of solutions containing 0.4 to 4.0 mgm. per cent) with each lot of unknowns.

m- and *p*-Aminohippuric acids, *p*-aminophenaceturic acid, *p*-aminophenylsuccinic acid, *p*-aminobenzoic acid, and *p*-aminomandelic acid. All these compounds couple satisfactorily in Bratton and Marshall's (18) method for sulfanilamide. The slight modifications presented here have, however, proved to be practically advantageous.

(a) 1.2 N HCl

(b) 100 mgm. per cent NaNO_2

(c) 500 mgm. per cent ammonium sulfamate

(d) 100 mgm. per cent *N*-(1-naphyl)ethylenediamine dihydrochloride

To 10 ml. of plasma filtrate or diluted urine add 2 ml. of (a) and 1 ml. of (b), and mix the solution; not before 3 minutes and not after 5 minutes, add 1 ml. of (c) and mix; between 3 and 5 minutes later, add 1 ml. of (d) and mix. A blank made of 10 ml. of water treated in the same manner is used to set the colorimeter at 100. Solutions are read at any time after 10 minutes, using a 540 mu. filter.

With *p*-aminohippuric acid, color development is maximal in 5 minutes, and the color is quite stable.

A standard curve is set up with 10 ml. samples of solution ranging in concentration from 0.02 to 0.25 mgm. per cent of *free* *p*-aminohippuric acid, the acid being dried if necessary at 90° C. overnight before preparation of the stock solution. The sodium salt is too hygroscopic to use as a standard. The standard curve is quite reproducible and needs to be rechecked only on rare occasions. A separate curve, however, should be prepared for each colorimeter.

The NaNO_2 must be prepared fresh every 3 days, the ammonium sulfamate every 2 weeks; the coupling reagent (d) lasts indefinitely if kept in a dark bottle and in the refrigerator. *p*-Aminohippuric acid is stable in plasma filtrates and urines if kept in the ice box.

(Bratton and Marshall (18) recommend a trichloroacetic acid filtrate for sulfanilamide, but we have found this filtrate to be quite unreliable for *p*-aminohippuric acid. Although perfect recoveries may be obtained, the coupling reaction frequently and inexplicably fails to go forward quantitatively or reproducibly. No such difficulty has ever been encountered with the cadmium sulfate filtrate, which gives excellent checks in duplicate determinations on independent precipitations.)

The chromogenic power of various compounds when corrected for molecular weight (but not specially dried) and the time required for maximal color development are as follows: *p*-aminohippuric, 100 per cent < 10 minutes; *m*-aminohippuric, 96 per cent, 30 minutes; *p*-aminobenzoic, 97 per cent, 20 minutes; *p*-aminomandelic, 90 per cent, 60 minutes; *p*-aminophenylsuccinic, 75 per cent, 90 minutes; *p*-aminophenaceturic, 83 per cent, 5 hours. In all cases, the color is quite stable.

p-Acetaminohippuric acid. Hydrolysis to *p*-aminohippuric acid is carried out by the addition of 2 ml. of

1.2 N HCl to 10 ml. of filtrate or diluted urine, contained in graduated narrow test tubes or Folin sugar tubes. After thorough mixing, the tubes are closed with glass tears and heated in a boiling water bath for 1 hour. The tubes are then cooled and the volume made up to 12.5 ml. and the p-aminohippuric acid content determined as above. If read against the standard p-aminohippuric acid curve, prepared as above, correction must be made for the increased total volume after the addition of coupling reagents, *i.e.*, the result must be multiplied by $12.5 \div 5/10 \div 5$ or 1.17.

Recoveries by this method are imperfect, some of the chromogen being destroyed, perhaps by oxidation. (Similar imperfect recovery after hydrolysis of p-acetylaminobenzoic acid is reported by Doisy and Westerfeld (19).) It is therefore preferable to prepare a standard curve by treating knowns in the manner of unknowns. Since the extent of loss appears to be fairly uniform in filtrates and urines, no large error is introduced in clearance determination.

Conjugated p-aminohippuric acid. Since absolute recoveries were essential in answering the question of possible conjugation in the body, the following method, which appears to be wholly satisfactory, was developed later in the investigation: 2 ml. of 10 N HCl are added to suitable aliquots of plasma filtrate or diluted urine in 50 ml. volumetric flasks, made up to volume and mixed. The solution in the neck is covered with about 1 ml. of toluene. The flasks are placed in an oven at approximately 96° C. for 3½ hours. After cooling, 10 ml. of this solution are taken for analysis by the p-aminohippuric acid method. p-Aminohippuric acid when treated in this manner gives 100 per cent recovery, as compared with analysis of an unheated solution. For this reason, we believe that recovery of conjugated material is essentially complete. (A presumptively pure sample of p-acetylaminosulfanilamide gave 95 per cent recovery by this method.)

Diodrast was determined by Alpert's method (20). In man, recovery in the 1:15 cadmium sulfate filtrate averaged 94 per cent (*cf.* Alpert's paper) but in dog recovery averaged so close to 100 per cent that no correction has been made. *Hippuran* and *iopax* were determined by the fusion method (2). Inulin was used in quantities sufficient to give plasma concentrations of 80 to 150 mgm. per cent and determined by the macro-method (2) on a ZnSO₄-NaOH filtrate (21). Mannitol was determined by the method of Smith, Finkelstein, and Smith (21).

2-Pyridone-1-acetic acid and *cinnamoylglycine* were determined in cadmium sulfate filtrates by a Hilger medium two-beam ultraviolet spectrograph with Spekker attachment, using standard procedures. We are indebted to Dr. Rudolph Naumann for these determinations.

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GELATIN AS A PLASMA SUBSTITUTE: THE EFFECTS OF INTRAVENOUS INFUSION OF GELATIN ON CARDIAC OUTPUT AND OTHER ASPECTS OF THE CIRCULATION OF NORMAL PERSONS, OF CHRONICALLY ILL PATIENTS, AND OF NORMAL VOLUNTEERS SUBJECTED TO LARGE HEMORRHAGE

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Recent experimental and clinical studies have demonstrated that especially prepared ossein gelatin, administered intravenously, is a safe and effective plasma substitute for use in the treatment of shock and is useful in certain hypoproteinemic states in man (1 to 3). Other investigators have suggested that gelatin shows some promise as a source of nutritional protein for intravenous administration (4, 5). It has seemed important to us that these studies should be extended to include observations on the dynamics of the circulation as affected by the intravenous infusion of the gelatin colloid in man.

There are available a number of reports on the circulatory effects of the intravenous infusion of saline or glucose solutions (6 to 11) and of colloidal solutions such as whole blood, plasma, or acacia (12 to 15). We have observed the changes in pulse rate, blood pressure, blood volume, venous pressure, cardiac output, hematocrit, and plasma protein produced by the intravenous infusion of gelatin in normal subjects, chronically ill patients, and volunteers subjected to experimental hemorrhage. The effect of hemorrhage *per se* proved to be of particular interest and has been the subject of a separate paper prepared in collaboration with others who were concurrently engaged in the study of this problem (16, 17).

Of the work reported here, the clinical studies carried out on normal persons, on patients, and on the volunteers after hemorrhage, recorded in Tables II, III, and IV, were made by Doctors Fletcher and Hardy. Unfortunately, these authors were inducted into the armed forces before the study was quite completed. The chemical analyses were all performed by Dr. Riegel. The estimations of blood volume were all made by Dr.

Koop. The special study on venous pressure after gelatin infusion was carried out by Dr. Koop with the kind assistance of Dr. R. L. Mayock. We are indebted to Dr. Isaac Starr for assistance in the final preparation of the manuscript.

MATERIAL AND METHODS

The gelatin used in this study was specially prepared for intravenous use by the hydrolysis of alkali-treated long bone collagen under carefully standardized conditions.¹ The material was made up in a 6 per cent solution in 0.9 per cent saline and autoclaved in 500 ml. containers at 15 pounds pressure for 20 minutes.

It should be pointed out that several different preparations of gelatin are now under investigation. Since they vary in respect to size of gelatin molecules and colloid osmotic pressure, and, therefore, in their physiological effects, the data presented herewith are to be considered only in relation to the specific preparation described. The gelatin solution used in these studies has been designated P-20.

The normal subjects studied were healthy male volunteers between the ages of 18 and 50 years. A group of 5 chronically ill patients, ranging in age from 14 to 65, were also selected for a study from the wards of the University Hospital.

In the hemorrhage experiments, venous blood was drawn under mild suction through a large bore needle into a graduated collecting bottle.

Estimates of the cardiac output were made by the use of the ballistocardiograph, the results being calculated according to the area method (18). The results are reported as percentage deviation from an empirical average normal value, the normal limits being ± 22 per cent. These studies were carried out in the laboratory of the Hartzell Research Department of Therapeutics through the courtesy of Dr. Isaac Starr.

Specimens of venous blood were drawn with a minimum of stasis at suitable intervals for the determination

¹ Prepared and supplied by Dr. D. Tourtellotte, Charles B. Knox Gelatin Company, Johnstown, N. Y.

of the hematocrit and the concentration of plasma protein and gelatin. Hematocrit determinations were made in duplicate on oxalated blood in Van Allen tubes centrifuged at 2500 r.p.m. for 30 minutes. Analyses for plasma protein and gelatin were carried out by differential precipitation and nitrogen determination by a Kjeldahl semi-micro technic.

Estimates of the blood volume were made from the dilution of the dye T1824 by a technic modified from that of Gibson and Evans (19).

Venous pressure was determined by an adaptation of the direct method of Moritz and Tabora (20) using a manometer filled with citrate solution and connected by a 3-way stop-cock to the infusion apparatus. Subjects lay supine, and the zero point selected was one-half of the depth of the chest, measured from the angle of Louis to the surface of the bed. No reading was accepted unless the respiratory variations could be clearly seen.

For some time, a single needle inserted in a vein was used both to measure venous pressure and to introduce the gelatin, and this is the technic usually adopted. Our experiments had been nearly completed before the hazard of this procedure was realized.

In every estimate of venous pressure by the technic of filling a burette and allowing the fluid to run into the vein as far as it will, the final reading should be corrected for capillarity and resistance in the tubing and needle. Thus, when the needle of our venous pressure apparatus is immersed in a beaker of water and the stop-cock opened, the column in the burette always comes to rest above the water level in the beaker. The amount of this difference, usually about 1 cm., was subtracted from the burette readings to estimate venous pressure. Eventually we realized that the viscous gelatin by sticking in the needle might reduce its lumen and so increase the magnitude of this correction for resistance. This might, perhaps, account for the apparent increase in venous pressure found after gelatin infusions by ourselves and others. Therefore, experiments were designed to test the matter.

TABLE I

Estimation of venous pressure in arm veins before and after gelatin infusion, using the needle through which gelatin had been injected and also a clean needle of similar size

Pa- tient	Before gelatin infusion			After gelatin infusion				
	Blood vol- ume	Venous pressure		Blood vol- ume	Venous pressure			
					"Gelatin" needle		"Clean" needle	
		Right arm	Left arm		Right arm	Left arm	Right arm	Left arm
	ml.	cm. H ₂ O	cm. H ₂ O	ml.	cm. H ₂ O	cm. H ₂ O	cm. H ₂ O	cm. H ₂ O
E. M.	2638	10.3	7.6	3040	9.3	8.1	9.6	9.0
L. A.	2638	6.5	3.7	3590	8.1	6.6	6.9	7.0
T. O.		3.2	6.4		4.8	7.9	4.6	7.9

It was easy to demonstrate that such an error might occur. The apparatus used for venous pressure was employed, the 20 gauge needle being immersed in a beaker of water at room temperature (24° C.). A series of readings showed that the burette column came to rest 0.9, 0.9, 0.8, 0.8, and 0.9 cm. above the fluid level in the beaker. Then, mimicking the technic of intravenous injection, 250 ml. of gelatin was passed through the needle. After this the column in the burette came to rest 3.5, 3.2, 2.9, 3.2, 3.0, and 3.0 cm. above the water level in the beaker. Cleaning the needle restored the original resistance. The failure to correct for such a change in resistance would account for the increased value for venous pressure after gelatin, recorded in several, but by no means all, of our experiments.

However, the difference in resistance caused by gelatin in the example cited was the largest we encountered in many similar experiments with 20 or 19 gauge needles, the size we employed for venipuncture. Furthermore, if the needles were kept at body temperature by immersion in water at 37° C., perfusing gelatin through them had no noteworthy effect on the resistance of the system. Also, the results obtained at room temperature were most irregular and increases in resistance after gelatin varied from 0 to 2.2 cm. Obviously, the result depends on the many factors affecting the viscosity which varies with the type of gelatin used, the temperature, the degree of dilution with saline used to fill the tubing, and especially with the cleanliness of the needle.

To study the matter further, venous pressures were measured in both arms of 3 patients, before and after infusions of gelatin. After the infusion, the estimate was made by using the needle through which the gelatin had been administered and also a second clean needle of similar gauge. The results, Table I, emphasize the variability of pressures measured in different peripheral veins. However, the averages showed that errors which could be attributed to clogging the needle with gelatin were negligible, so we accepted our results made with the original technic.

RESULTS

A. Intravenous infusions of gelatin in normal male subjects

The effects of 5 intravenous infusions of large amounts of gelatin were observed in 3 healthy male subjects. The data are presented in Table II, and Figure 1 shows the averages of the results obtained.

The 2 subjects who received gelatin twice experienced on each occasion a slight feeling of tightness in the chest during the infusion and also a throbbing headache which was most marked 3 or 4 hours after the infusion had been completed. This gradually disappeared during the subsequent

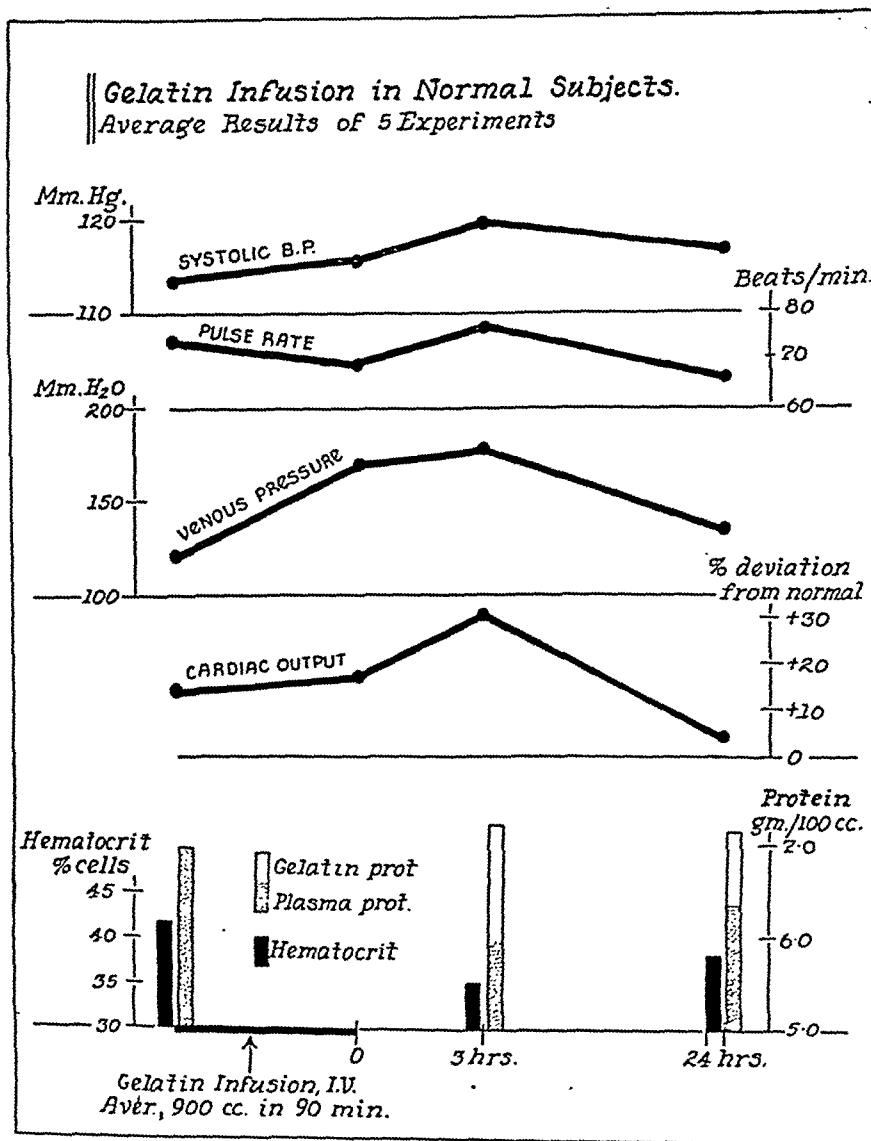


FIG. 1. AVERAGE RESULTS OBTAINED IN 5 NORMAL SUBJECTS BEFORE AND FOR 24 HOURS AFTER A LARGE INFUSION OF GELATIN INTRAVENOUSLY

24 hours. There were no other untoward symptoms of any kind.

The results indicate that the pulse rate and blood pressure of healthy persons were little changed by a gelatin infusion. The average venous pressure was slightly increased by the infusion. The average cardiac output was not increased significantly immediately after the infusion but it became so 3 hours later, being well above normal limits at that time. This increase was not found 24 hours later. The fact that the last cardiac output was below the initial estimate is not to be attributed to the gelatin, for apprehension

increases the cardiac output before most experimental procedures, and this passes off as the conclusion approaches.

Hematocrit and plasma protein concentration diminished markedly after the infusion. Indeed, the plasma proteins never, and the hematocrit only once, regained their initial values in 24 hours.

B. Intravenous infusion of gelatin in chronically ill patients

Somewhat less complete studies of the circulation were made during 6 infusions in 5 chronically ill patients of the type to whom gelatin might be

TABLE IV
Intravenous infusion of gelatin after acute hemorrhage

Subject	Physical data			Volume bled		Volume 6 per cent gelatin injected	Time of observations	Pulse rate	Blood pressure	Peripheral venous pressure	Cardiac output, deviation from average normal	Plasma protein	Plasma gelatin	Hematocrit
	Weight	Height	Surface area	Amount	Percentage of blood volume estimated from wt.									
	kgm.	cm.	sq. m.	ml.	per cent	ml.			mm. Hg	mm. H ₂ O	per cent	grams per 100 ml.	grams per 100 ml.	percentage of cells
T. K.	84	175	2.00	1200	20	840	Initial After hemorrhage After gelatin	74 92 78	96/70 84/64 96/66	112 120 140	- 4 - 9 - 9	6.20 5.92 4.30	1.23	42.0 39.5 28.5
J. R.	82	185	2.06	1065	17	710	Initial After hemorrhage After gelatin	66 69 72	128/68 130/70 130/70	146 146 150	+13 - 2 +26	6.36 5.53 4.90	1.00	42.0 39.0 33.5
C. M.	61	170	1.72	900	18	600	Initial After hemorrhage After gelatin	71 53 62	120/78 58/40 110/60	164 124 164	+ 4 -11 +13	6.98 5.13	0.87	36.0 28.0
B. B.	68	173	1.81	1020	19	680	Initial After hemorrhage After gelatin	77 77 69	114/60 100/60 108/58		+65 +30 +43			36.5 35.0 29.0
E. T.*	75	170	1.86	1050	19	750	Initial After hemorrhage After gelatin	89 45 76	130/80 120/70		+22 -39 - 2			48.5 45.5 37.0
H. G.	69	173	1.81	1000	19	710	Initial After hemorrhage After gelatin	71 58 66	118/70 68/34 108/60	140 95 146	+17 0 +13			43.0 39.0 33.0
R. C.	89	173	2.03	1020	17	680	Initial After hemorrhage After gelatin	67 71 77	120/60 120/80 120/60	140 120	-17 -13 +13			39.5 37.0 29.5
L. J.	73	180	1.92	1090	19	730	Initial After hemorrhage After gelatin	112 111 94	150/80 130/80 134/66	170 140 175	+48 +48 +35			40.0 38.0 30.5
W. C.†	76	178	1.91	920	16	760	Initial After hemorrhage After gelatin	74 47 78	130/80 70/40 130/70		+17 -17 +13			41.0 39.5 30.0

* Standing B.P. 108/80.

† Standing B.P. 114/78.

hemodilution due to the saline infusion, showed no further hemodilution and, in fact, some hemocentration during the next 12 hours. Subject T. N., receiving gelatin replacement, showed the greatest reduction of hematocrit and plasma protein. The latter value found in the estimation made 6 hours after the start, is so low that we suspect an error although we were unable to find it.

After the hemorrhage, as long as the 3 subjects lay recumbent they were free of symptoms and differences in their pulse rate, blood pressure, and cardiac output were not conspicuous; but, as soon as the subjects arose, the differences were very conspicuous. The subject who had received gelatin was able to stand and walk without symptoms on his first attempt, 3 hours after hemorrhage, and had no trouble thereafter. In contrast, dizziness and a marked fall in blood pressure on

arising persisted in the other 2 subjects for at least 12 hours, although in A. F., these symptoms were improved temporarily after the saline infusion.

After the rest of the experiments had been completed, to assist in the interpretation of the changes in hematocrit and plasma protein values found after gelatin, one of the authors (Koop) made estimates of blood volume before and after gelatin injection. Patient P. O. was chosen because he was judged to be well hydrated, having received a total of over 3000 ml. of fluid daily for 4 days, part by intravenous administration and the remainder through a gastrostomy. Patient J. O. was chosen because he was obviously dehydrated, as the only fluid he had received in the last 36 hours was 500 ml. of tap water by rectum. During this period, he had sweated profusely at operation, and at the time of the test he was extremely

Time

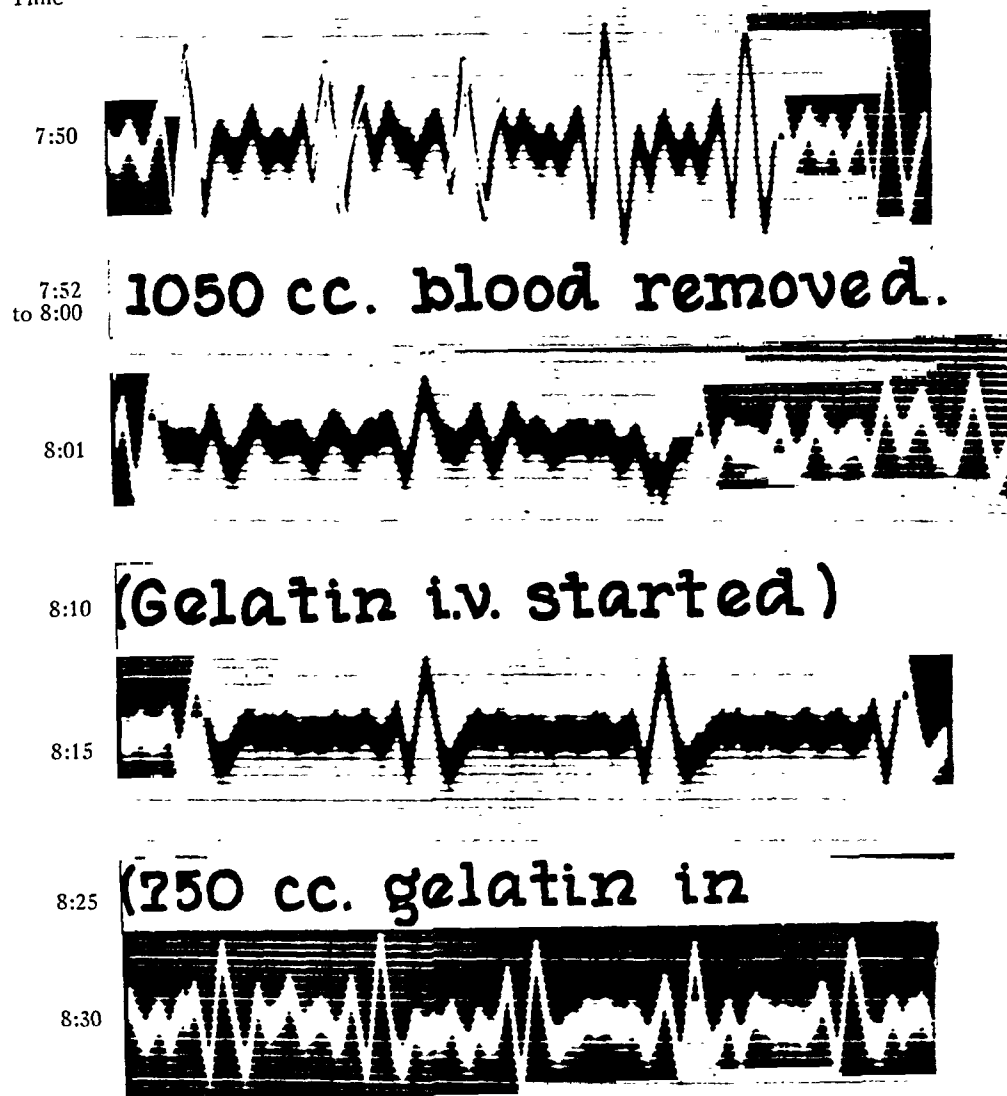


FIG. 2. BALLISTOCARDIOGRAMS BEFORE AND AFTER A LARGE HEMORRHAGE

The reproduction is actual size. Subject E. T., age 38, 5 feet 7 inches, 165 pounds.

At 7.50, the B. P. 130/80, the pulse rate 89, the cardiac output + 22 per cent above average normal (normal limits ± 22 per cent).

After the hemorrhage, at 8.01, the subject was sweating, pale, restless, and manifested greatly increased respiration. He complained that his vision was dim. Blood pressure could not be obtained. The pulse rate was 45. The ballistocardiogram is distorted by the subject's restless movements and by impacts from respiratory movements. Selecting the undistorted complexes the cardiac output was calculated to be - 40 per cent.

At 8.15, after gelatin had been started, the symptoms were much improved, B. P. 50/30, pulse 52, cardiac output - 30 per cent.

At 8.30, after completion of the infusion, the subject had no symptoms, the B. P. 110/72, the pulse rate 77, the cardiac output did not deviate from average normal.

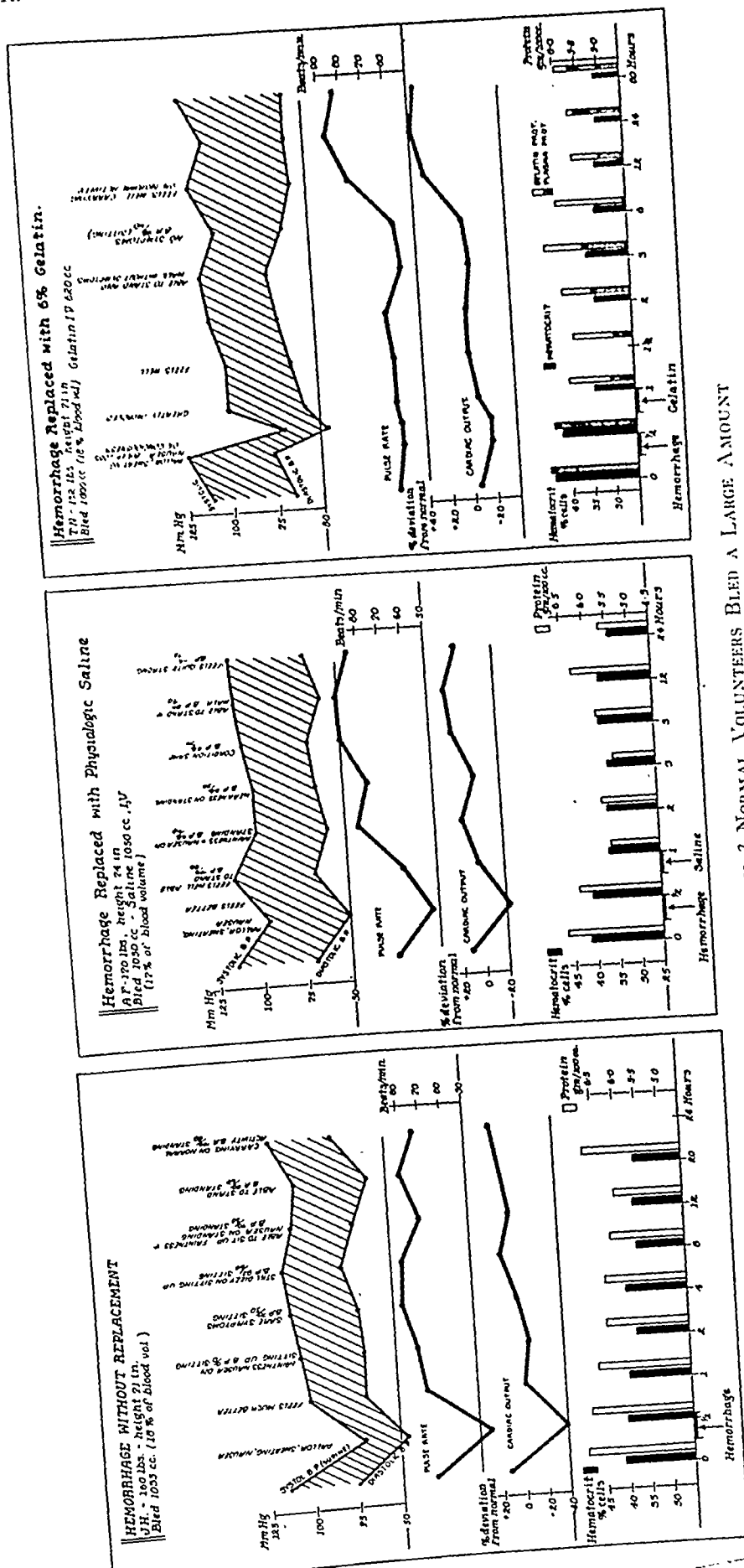


FIG. 3. RESULTS OBTAINED IN 3 NORMAL VOLUNTEERS BLED A LARGE AMOUNT

in the first, the blood lost was not replaced; in the second, it was replaced by saline solution; in the third, by gelatin.

thirsty. The other patients, convalescent and taking fluids *ad lib*, were judged to be normally hydrated. The results are recorded in Table V. They show that in the 4 patients who were well supplied with fluid the estimated increment of blood volume 3 hours after the end of the injection was larger, or but little less, than the amount injected. In the dehydrated patient, this increment 3 hours later was less than half the volume injected.

DISCUSSION

After the administration of gelatin solution intravenously, the plasma protein concentration and hematocrit diminish more than can be accounted for by the volume of fluid injected. This dilution slowly increases, reaching a maximum several hours after the injection. While some investigators (7) have believed that changes in plasma volume could be estimated from the hematocrit with reasonable accuracy, others (24, 25) have produced evidence that the errors in such an estimate were often great and that a similar estimate from changes in plasma protein concentration was also unsatisfactory. The frequent lack of concordance between the results of estimates of blood volume made under similar conditions by methods based on different physiological principles makes the accuracy of any method difficult to judge, but the effect of gelatin on blood volume seems fairly clear. In dogs, given far larger amounts of gelatin in relation to blood volume than those we employed, it was found (26) that blood volume, as estimated by T 1824, was markedly increased after the infusion, although the increment was generally somewhat less than the volume of the injected solution. In man, using the same dye, other authors (27) estimated that the increase of plasma volume, immediately after the injection of a gelatin, somewhat different from that we employed, averaged 58 per cent of the amount injected; but, thereafter, the blood volume continued to increase, the average maximal increment, 70 per cent of the amount injected, being found 4 hours afterwards. Results obtained in the Harrison Department of Research Surgery suggest that the small molecular gelatin used by these investigators (27) appears in the urine more rapidly than that employed in this

TABLE V

Plasma volumes calculated from dilution of T 1824 before and 3 hours after the intravenous injection of 500 ml. of 6 per cent gelatin solution

Patient	Weight	Before injection		After injection		Change in plasma volume
		Plasma volume	Hematocrit	Plasma volume	Hematocrit	
	kgm.	ml.	per cent	ml.	per cent	ml.
P. O.	43	1988	42.5	2785	29.1	695
S. E.	55	2280	44.8	2710	39.3	430
J. O.	64	2920	44.3	3160	42.5	240
E. M.	60	2638	48.4	3040	40.8	402
L. A.	64	2638	52.4	3590	45.1	952

study. Nevertheless, our results are in essential accord with theirs, the dilution reaching its maximum in the samples taken 3 or 4 hours after injection, at which time the hematocrit and plasma protein values could be accounted for by assuming a degree of dilution approximately equal to the volume of fluid injected. The average increase in blood volume, as measured by T 1824, is of this magnitude (Table V). Gelatin must be slowly lost from the blood stream, for it appears in the urine (1), but it also has the power of attracting fluid into the blood by its osmotic properties. Apparently, for the first few hours after injection, the second effect often predominates, and the volume added by injection may be maintained or even increased.

The results indicate that there is a small increase in venous pressure after gelatin infusion. Approximately equivalent increments were found by others (23) after infusion of similar amounts of serum.

The cardiac output increased significantly after the gelatin infusion in 3 of the 5 normal subjects (Table II), and this increase was much more conspicuous in all the 6 chronically ill patients (Table III). Even larger percentage increases of cardiac output were found (26) to follow gelatin infusion in dogs, but the amount of solution injected was much larger in proportion to the blood volume than was used in this study, so these results are concordant with ours.

In the case of the 3 subjects who were bled, one treated with gelatin, one with saline, and the other left untreated as a control, we were repeating on man a type of experiment performed in animals in many laboratories for the benefit of

EFFECT OF METHYL TESTOSTERONE ON URINARY 17-KETOSTEROIDS OF ADRENAL ORIGIN^{1, 2, 3}

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Urinary 17-ketosteroids or their precursors are thought to arise from the adrenal cortices and the testes (1). Testosterone propionate is partially excreted as a 17-ketosteroid (2); on the other hand, 17-methyl testosterone is not excreted as such (3 to 5). It follows that methyl testosterone in contradistinction to testosterone propionate, may be used to study the effect of a testosterone compound on the endogenous production of urinary 17-ketosteroids. The present paper is primarily concerned with the effect of methyl testosterone on the urinary 17-ketosteroids of adrenal origin.

It is essential that such studies be carried out on individuals without functioning testicular tissue, since it is highly probable from animal experiments that testosterone inhibits its own endogenous production in the testis (6 to 8). It is difficult to obtain direct data in humans on the effect of testosterone on the urinary 17-ketosteroids of testicular origin. Thus, whereas the administration of methyl testosterone to a male without disease of the adrenals and testes induced a lowered 17-ketosteroid excretion (Figure 1), the entire effect may have been mediated through

the adrenal cortex. Actually to establish the point it would be necessary to give methyl testosterone to a male patient with Addison's disease. This was done (Figures 2 and 3), with suggestive but not conclusive results. Such an experiment runs into difficulties because the low initial level of 17-ketosteroid excretion makes the errors introduced by chromogens and other technical errors relatively more significant.

CLINICAL CASES

The influence of methyl testosterone on 17-ketosteroid excretion by individuals without testicular tissue was studied in 4 females with adrenal hyperplasia, 2 normal females, and 1 male with absence of functioning testicular tissue. Of the 4 patients with hyperplasia, 2 had Cushing's syndrome, and 2, the adrenogenital syndrome. Whereas hyperplasia of the adrenal cortices may occur in both of these conditions, it is the authors' belief that in the first of them there is primarily an over-production of a hormone concerned with carbohydrate metabolism (the so-called "sugar" or "S" hormone), while in the second there is an over-production of a hormone concerned with anabolism of protoplasm, and masculinization (the so-called "nitrogen" or "N" hormone) (9). In Cushing's syndrome there may be a compensatory over-production of "N" hormone as well (9). One "normal" female had no disease; the other had Paget's disease but was normal with respect to adrenal cortical function. The male patient had clinical evidence of hypopaydigism, a high excretion of follicle-stimulating hormone in the urine, a high 17-ketosteroid excretion, and, as demonstrated at exploration at the age of 8, bilateral rudimentary testes. Thus, the evidence for lack of testicular tissue includes not only the clinical evidence of hypopaydigism and the findings at operation but the high titer of follicle-stimulating hormone (11). The high 17-ketosteroid excretion in spite of the clinical picture of hypopaydigism presumably represents compensatory hyperplasia of the adrenal cortex, such as is known to occur in animals castrated at birth (12, 13). The findings are entirely dissimilar to those in the usual eunuchoid patient, where the follicle-stimulating hormone excretion in the urine is usually normal or low and the 17-ketosteroid is regularly low (14).

¹ The expenses of this investigation were defrayed in part by the Josiah Macy, Jr. Foundation and by a grant from the Committee on Endocrinology of the National Research Council. The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Massachusetts General Hospital.

² The authors wish to thank the Schering Corporation, Bloomfield, New Jersey, for the generous supplies of methyl testosterone (Oretan-M) and the Ayerst, McKenna and Harrison Company, New York, for the large amounts of chorionic gonadotropin (A.P.L.) used in these studies.

³ Read by title at the 36th annual meeting of the American Society for Clinical Investigation, May 8, 1944.

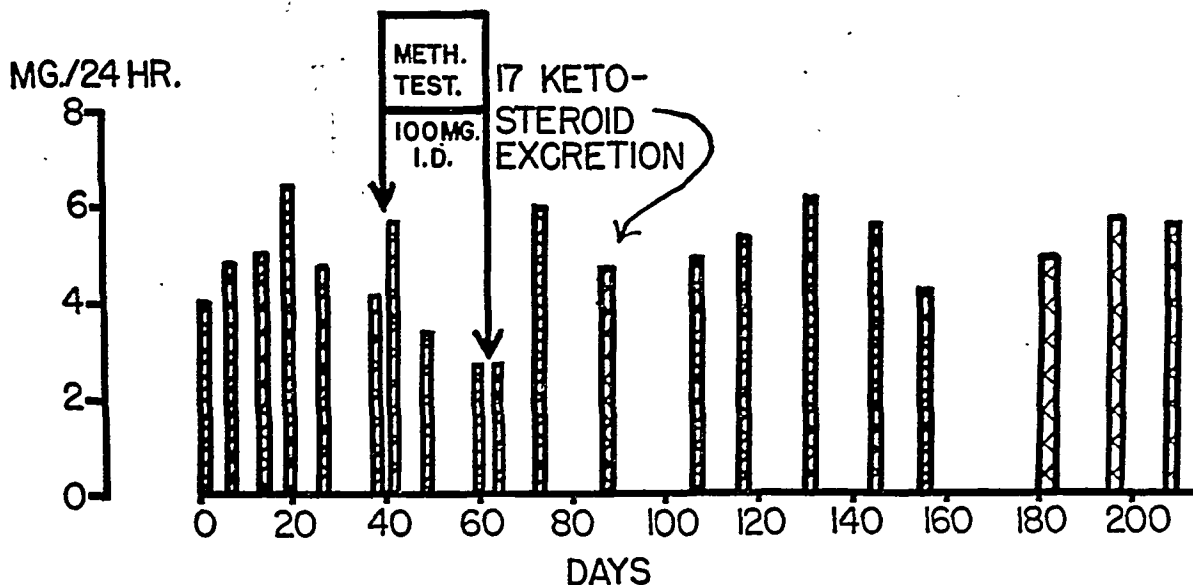


FIG. 1. EFFECT OF METHYL TESTOSTERONE THERAPY ON THE URINARY 17-KETOSTEROID EXCRETION IN A MAN WITH PAGET'S DISEASE OF BONE (OSTEITIS DEFORMANS)

In this patient (B. F., No. 36002, a male of 49 years), it is assumed that the functions of the adrenal cortices and testes are comparatively normal; however, the 17-ketosteroid excretion at the time of the experiment was low, probably because of debility (9). It will be noted that, although the control values were low (a mean of 4.8 mgm. per 24 hrs.) compared with the normal level in males (*circa* 14 mgm. per 24 hrs. (1)), with methyl testosterone therapy there was an orderly fall to a still lower level (a mean of 3.0 mgm. per 24 hrs.), and that with omission of the therapy the values rose to a level (a mean of 5.3 mgm. per 24 hrs.) approximating the pre-treatment level. The values for the first 6 days following a change in therapy were omitted in compiling the mean values. Note that the measurements were made on 24-hour collections.

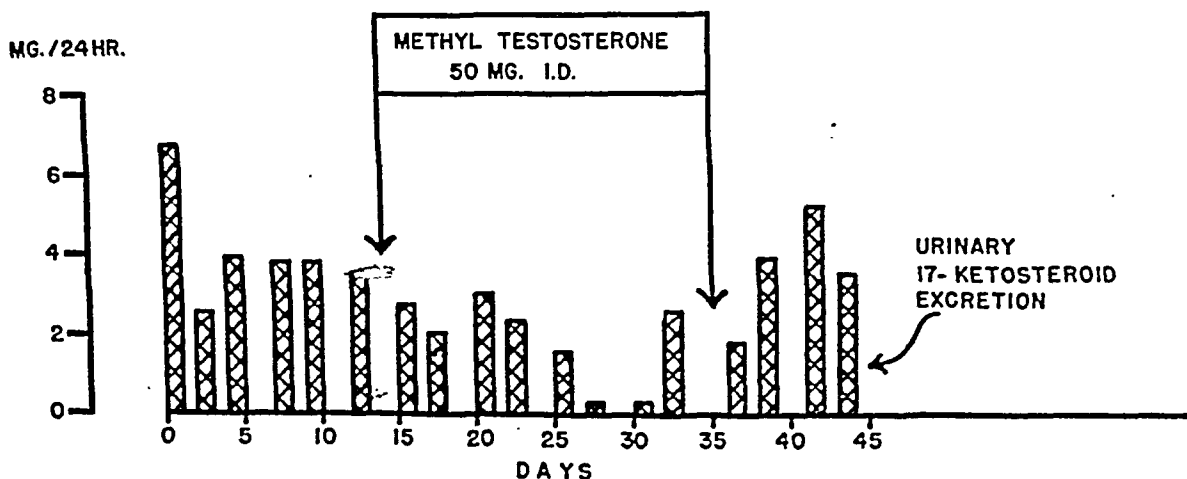


FIG. 2. EFFECT OF METHYL TESTOSTERONE ON THE URINARY 17-KETOSTEROID EXCRETION IN A MAN WITH ADDISON'S DISEASE

This patient, K. P., No. 369416, a male of 44 years, had tuberculosis of the adrenals. At the time of the experiment, the patient was taking no medication except 3 grams of sodium chloride by mouth daily. It will be noted that during the control period the 17-ketosteroid excretion was low (a mean of 4.0 mgm. per 24 hrs.) compared with the normal level in males (*circa* 14 mgm. per 24 hrs. (1)); that under methyl testosterone therapy the level was suggestively lower (a mean of 1.6 mgm. per 24 hrs.); that, when the drug was omitted, the level was increased (a mean of 4.3 mgm. per 24 hrs.). The values for the first 6 days following a change in therapy were omitted in compiling the mean values. Note that the measurements were made on 24-hour collections.

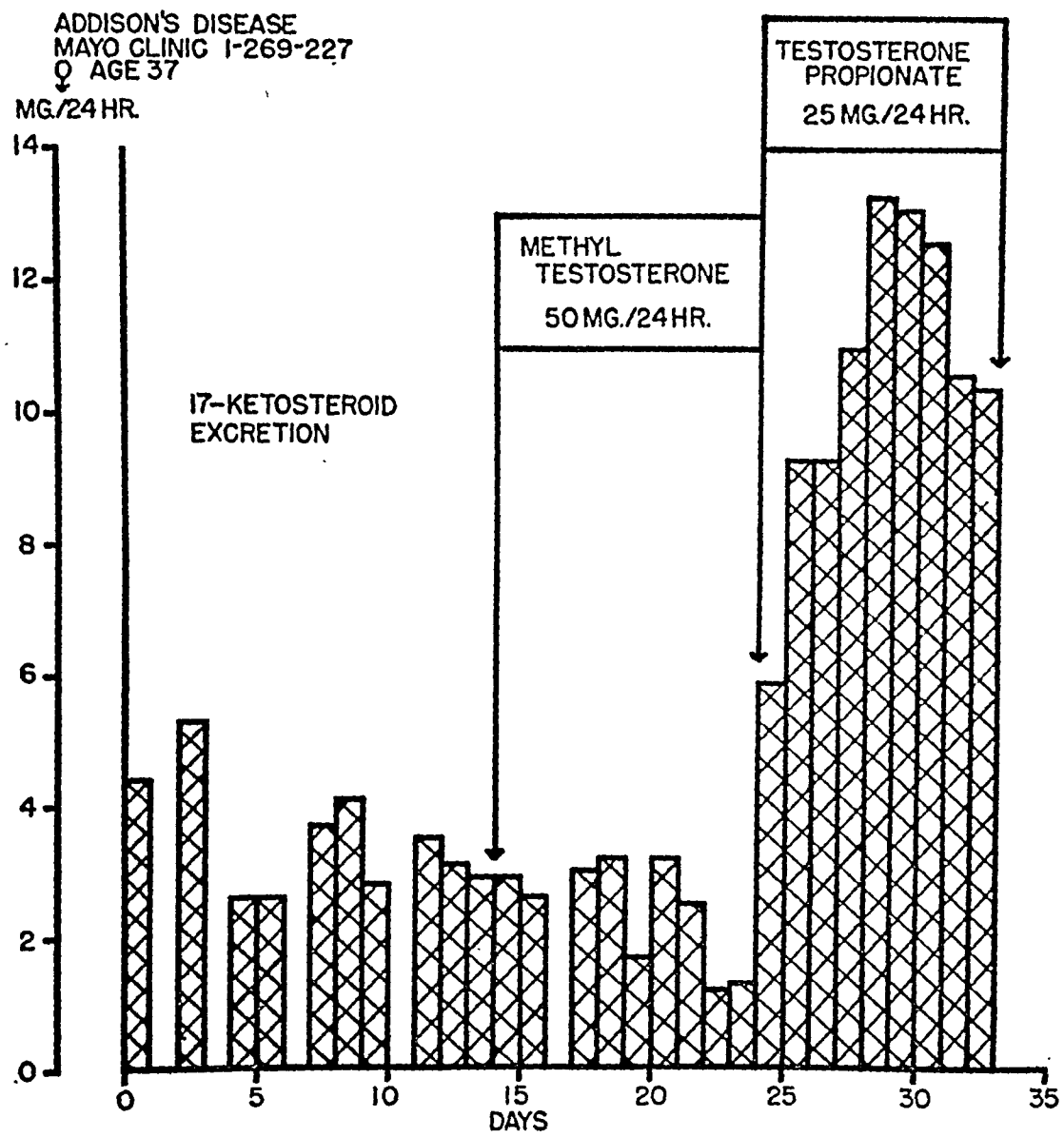


FIG. 3. EFFECT OF METHYL TESTOSTERONE ON THE URINARY 17-KETOSTEROID EXCRETION IN A MAN WITH ADDISON'S DISEASE

This patient, Mayo Clinic No. 1-269-227, a male of 37 years, had tuberculosis of the adrenals. The data on this patient were made available through the courtesy of Dr. Edwin J. Kepler, Rochester, Minnesota (10), to whom the authors are greatly indebted. During the first 6 days of the experiment, the patient received 10 grams of sodium chloride and 5 grams of sodium citrate by mouth daily; during the remainder of the experiment he was given 4 mgm. of desoxycorticosterone acetate each day intramuscularly. This therapy had no effect on the 17-ketosteroid excretion. It will be noted that during the control period the 17-ketosteroid excretion was low (a mean of 3.5 mgm. per 24 hrs.) compared with the normal level in males (*circa* 14 mgm. per 24 hrs. (1)), and that under methyl testosterone therapy the level was suggestively lower (a mean of 2.1 mgm. per 24 hrs.). The values for the first 6 days following a change in therapy were omitted in compiling the mean values. Note that the measurements were made on 24-hour collections.

METHODS

The 17-ketosteroid content of the urine was measured by a method based on a modification (15) of the Zimmermann reaction (16) that has been described previ-

ously (1). To correct the result for color introduced by chromogens, the reaction is read through a green and a violet filter, and a color correction equation applied. The method is accurate within 1 to 2 mgm. in repeated analyses of the same urine pool. Normal females ex-

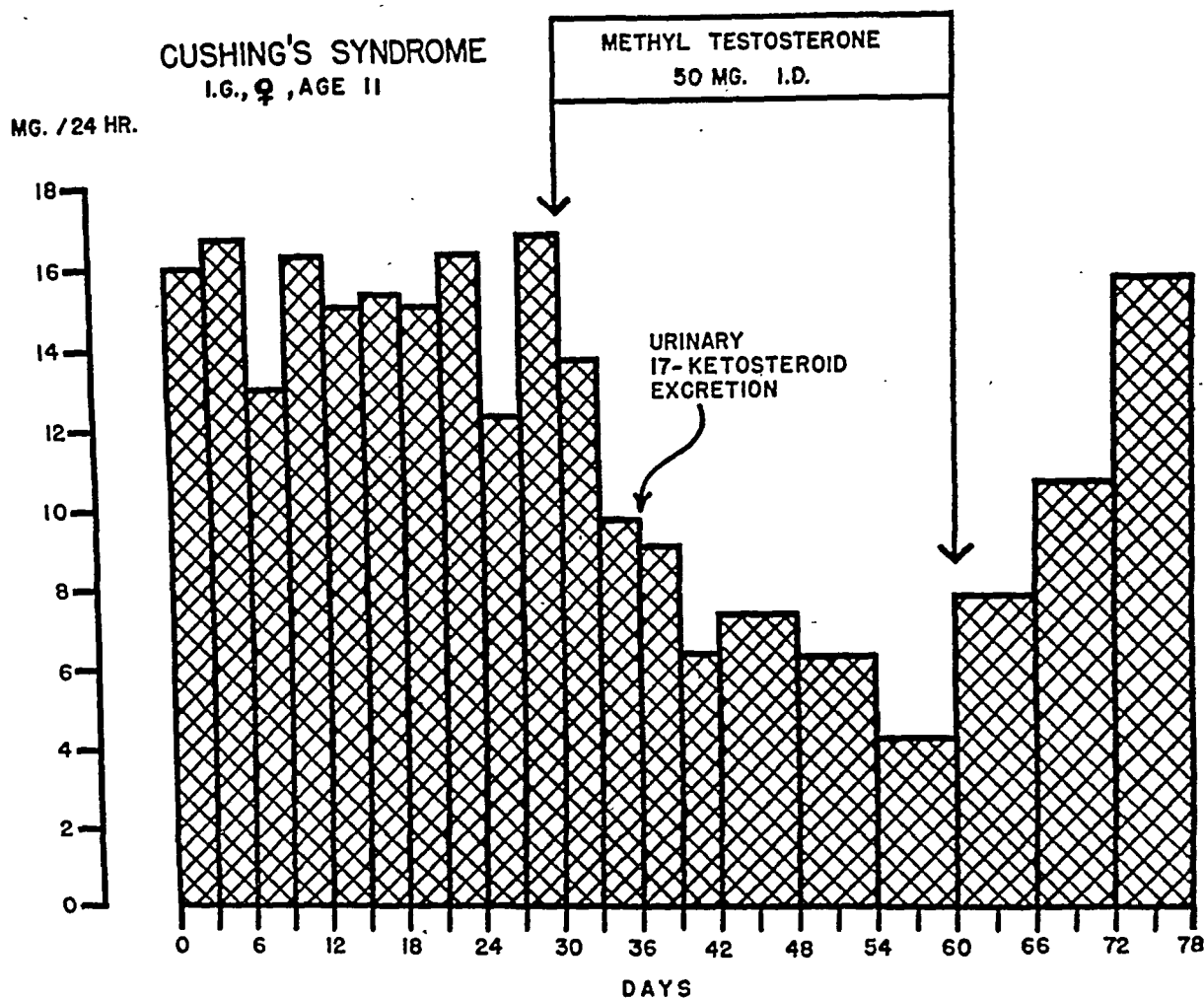


FIG. 4. EFFECT OF METHYL TESTOSTERONE THERAPY ON THE URINARY 17-KETOSTEROID EXCRETION IN CASE 1, A GIRL WITH CUSHING'S SYNDROME

For discussion, see text. Note that the measurements were made on 72-hour collections until day 42 and on 144-hour collections thereafter.

crete from 5 to 14 (average 9), normal males from 8 to 23 (average 14) mgm. of sterone per 24 hours (1).

The collection of urine specimens was rigidly supervised to insure accuracy. Cases 1, 3, and 6 were studied on the research ward, and their collections were handled by specially trained personnel. Most of the determinations in these 3 cases were made on aliquots of 72 to 144-hour pools. The other patients in this investigation collected their specimens at home and brought them personally to the laboratory; these individuals were instructed carefully in the method of collection and were questioned frequently to be certain that they followed the method accurately. Most of the determinations in these patients were on specimens that were obtained by pooling in the laboratory two accurately timed night collections. All of the results are reported in terms of amounts per 24 hours.

Methyl testosterone was administered to the patients by mouth in the form of 10 mgm. tablets.

RESULTS

In Case 1,⁴ I. G., No. 350260, a girl of 11 years with classical Cushing's syndrome, the effect of methyl testosterone was studied twice (Figures 4 and 5). It will be noted that during both control periods, the 17-ketosteroid excretion (*circa* 15 mgm. per 24 hours) was very high for her age (normal, *circa* 1 to 3 mgm. per 24 hours (17)) and quite constant; that under methyl testosterone therapy the excretion showed a very orderly fall to

⁴ Further clinical data are published elsewhere (9).

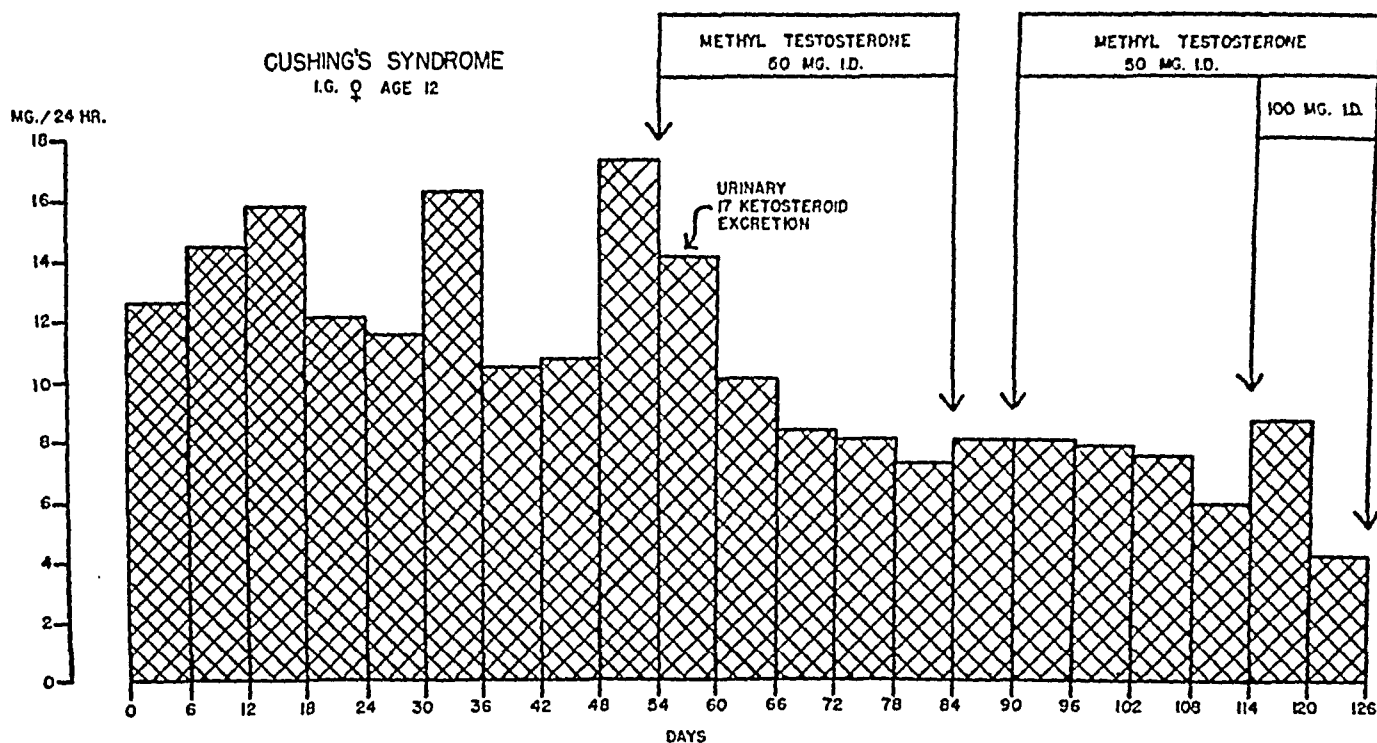


FIG. 5. CASE 1, REPEAT EXPERIMENT

For discussion, see text. Insulin, 25 to 100 units daily, was given intramuscularly from day 18 to day 42. Note that the measurements were made on 144-hour collections.

a considerably lower value (*circa* 4 mgm. per 24 hours); that, when the therapy was omitted, the excretion again rose.

In Case 2,⁵ R. B., No. 3397, a woman of 53 years, also suffering from Cushing's syndrome, it will be observed (Figure 6) that the average pre-treatment excretion (*circa* 18 mgm. per 24 hours) was high for an adult woman (normal *circa* 9 mgm. per 24 hours (1)) and very constant; that the excretion rose both with testosterone propionate and with dehydroisoandrosterone acetate; that under methyl testosterone therapy the excretion showed a gradual fall to a low value (*circa* 2 mgm. per 24 hours); and that, after discontinuation of methyl testosterone therapy, the excretion again rose to the pre-treatment level.

In Case 3, R. H., No. 401611, a girl of 6 years with congenital adrenogenital syndrome and pseudohermaphroditism, it will be noted (Figure 7) that during the control period, the 17-ketosteroid excretion (*circa* 15 mgm. per 24 hours) was very high for her age; that under methyl testosterone therapy the excretion showed a steady fall to a lower value (*circa* 10 mgm. per 24

hours); and that after the therapy was omitted the excretion definitely increased.

In Case 4,⁶ E. F., No. 240632, a woman of 24 years with a condition similar to Case 3, the average 17-ketosteroid excretion (Figure 8) (*circa* 45 mgm. per 24 hours) was very high during the control period; with methyl testosterone therapy the average level (*circa* 35 mgm. per 24 hours) was suggestively lower; and, when the drug was discontinued, the average excretion (*circa* 40 mgm. per 24 hours) was increased.

Case 5, A. S., a woman of 34 years, had no disease, and normal function of the adrenal cortex. It will be observed (Figure 9) that during the control period the average 17-ketosteroid excretion (*circa* 11.5 mgm. per 24 hours) was slightly above the average for an adult woman (normal, *circa* 9 mgm. per 24 hours (1)); that during the administration of 20 mgm. of methyl testosterone daily, the 17-ketosteroid excretion tended to be lower; that during the administration of 40 mgm. of methyl testosterone daily, the excretion showed a gradual fall to definitely low values (*circa* 6 mgm. per 24 hours); and that after discontinua-

⁵ Further clinical data are published elsewhere (1, 9, 18).

⁶ Further clinical data are published elsewhere (1).

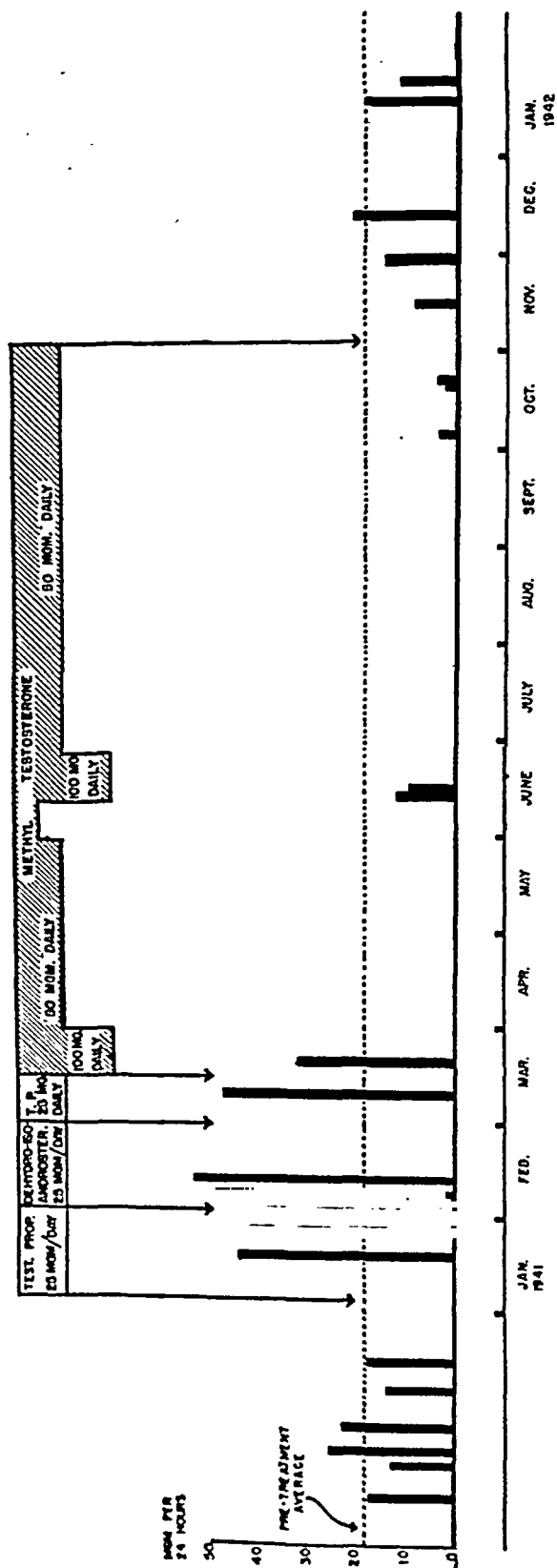


FIG. 6. EFFECT OF TESTOSTERONE PROPIONATE, DEHYDROISANDROSTERONE ACETATE, AND METHYL TESTOSTERONE THERAPY ON THE URINARY 17-KETOSTEROID EXCRETION IN CASE 2, A WOMAN, AGE 53 YEARS, WITH CUSHING'S SYNDROME

For discussion, see text. Note that the measurements were made on 16- to 24-hour collections.

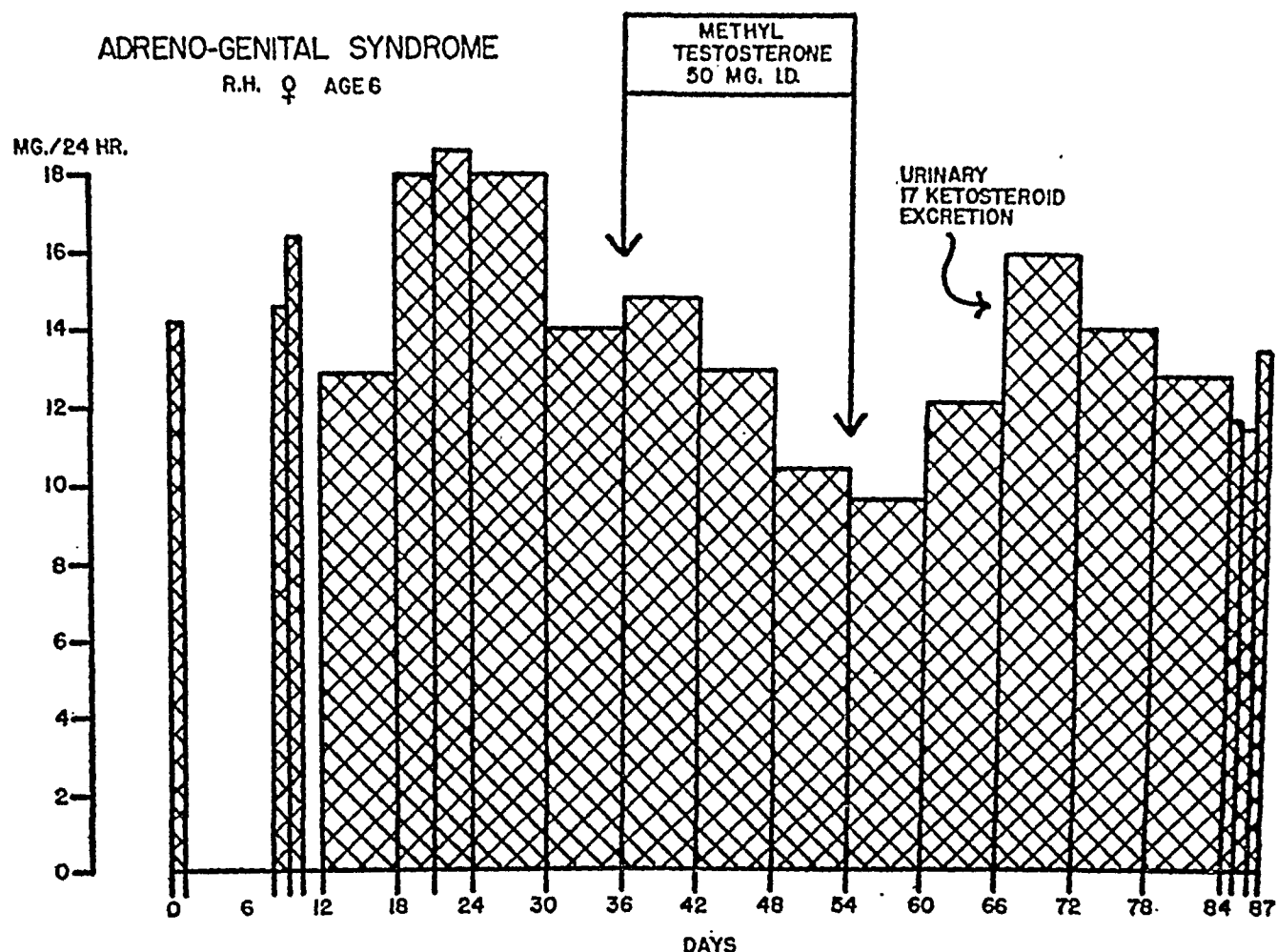


FIG. 7. EFFECT OF METHYL TESTOSTERONE THERAPY ON THE URINARY 17-KETOSTEROID EXCRETION IN CASE 3, A GIRL WITH ADRENOGENITAL SYNDROME

For discussion, see text. Desoxycorticosterone acetate, 10 mgm. daily, was given intramuscularly from day 66 to day 86. This therapy had no effect on the 17-ketosteroid excretion. Note that the measurements were made on 24-hour collections for the first 3 and the last 3 determinations, on 72-hour collections for days 18 to 24, and otherwise on 144-hour collections.

tion of methyl testosterone therapy, the excretion again rose to the pre-treatment level.

Case 6, F. S., No. 424919, a woman of 35 years with Paget's disease (osteitis deformans), is assumed to have normal function of the adrenal cortex. It will be seen (Figure 10) that, during the control period, the 17-ketosteroid excretion (*circa* 8 mgm. per 24 hours) was approximately average for an adult woman (normal *circa* 9 mgm. per 24 hours (1)); and that under methyl testosterone therapy the level (*circa* 6 mgm. per 24 hours) was moderately but hardly significantly lower.

Case 7, F. B., No. 84187, a man of 27, had before any specific treatment a high voice, small larynx, no beard, rudimentary prostate, absence

of testes in scrotum, moderately well developed phallus, axillary and pubic hair absent, scant hair on extremities, absence of recession of hair in temporal regions, and obesity. Bilateral rudimentary testes were found at operation at age of 8. The pituitary gonadotropic (follicle-stimulating) hormone in the urine (between 567 and 868 mouse units per 24 hours) was very high compared with the normal (less than 104 mouse units per 24 hours (19)). The excretion of 17-ketosteroids (*circa* 16 to 33 mgm. per 24 hours) was also high. It will be noted (Figure 11), that two experiments were carried out on this patient with different doses of methyl testosterone. The control values (*circa* 24 mgm. per 24 hours) were high; during the administration of 100 mgm. of

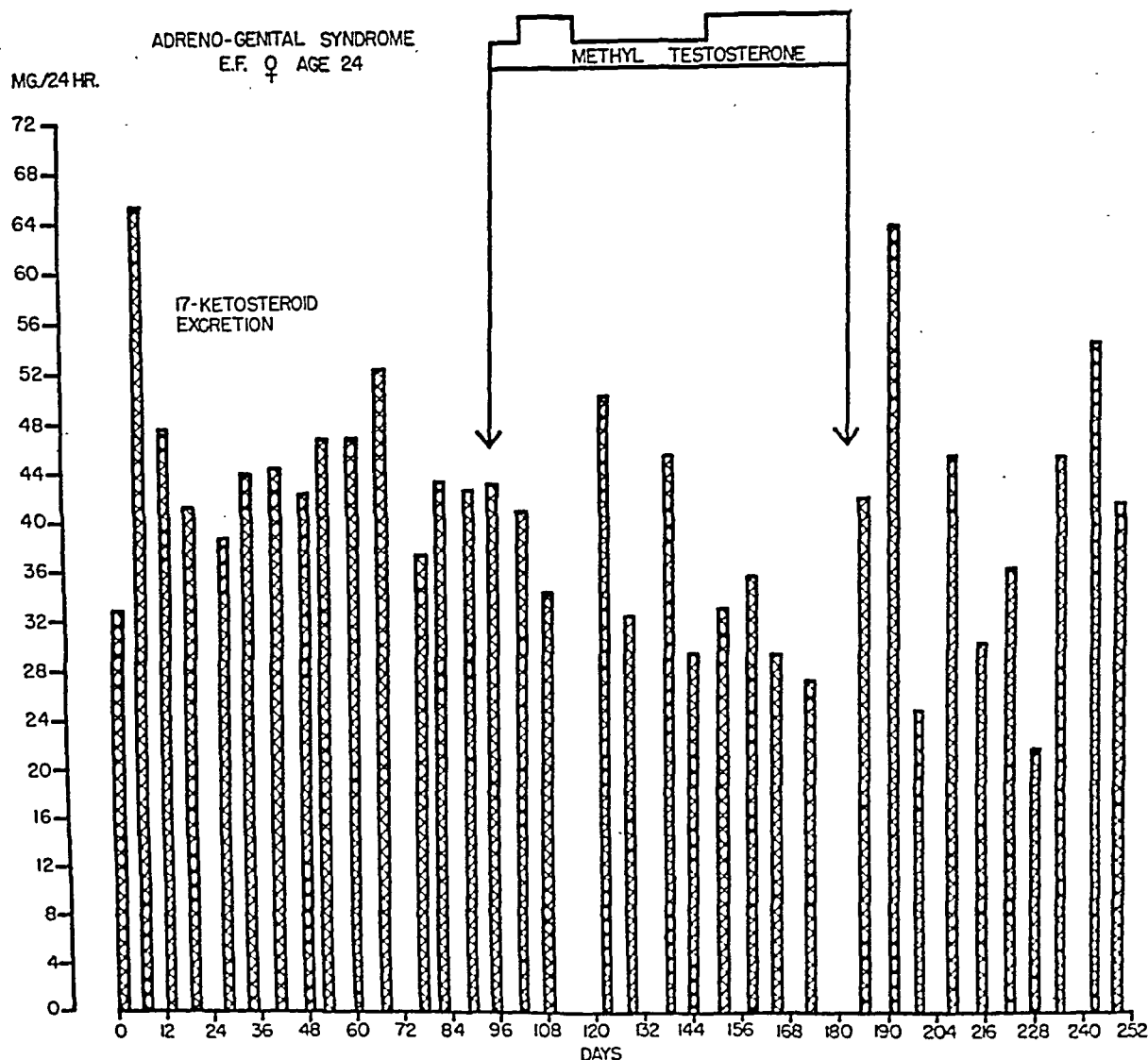


FIG. 8. EFFECT OF METHYL TESTOSTERONE THERAPY ON THE URINARY 17-KETOSTEROID EXCRETION IN CASE 4, A WOMAN WITH ADRENOGENITAL SYNDROME

For discussion, see text. Note that the measurements were made on 24-hour collections.

methyl testosterone daily there was a fall to a lower level (*circa* 19 mgm. per 24 hours); during the 14 days after the cessation of therapy, the level (*circa* 18 mgm. per 24 hours) continued low. This experiment was considered inconclusive and so was repeated with larger doses. During the administration of 200 mgm. of methyl testosterone daily, the 17-ketosteroid excretion (*circa* 10 mgm. per 24 hours) was definitely lower than the initial values (*circa* 24 mgm. per 24 hours) or the intervening control values (*circa* 18 mgm. per 24 hours); from the 14th to 27th day following ces-

sation of treatment, the level (*circa* 18 mgm. per 24 hours) was significantly higher. It will be noted in Figure 11 that chorionic gonadotropin (A.P.L.) was administered on two occasions. Although this therapy may have induced a transitory rise in the 17-ketosteroid excretion, this possibility has been ignored in compiling the mean values obtained during methyl testosterone therapy (Table I).

The data from these studies are summarized in Table I. Since there is a delay in the onset and in the cessation of effect following the adminis-

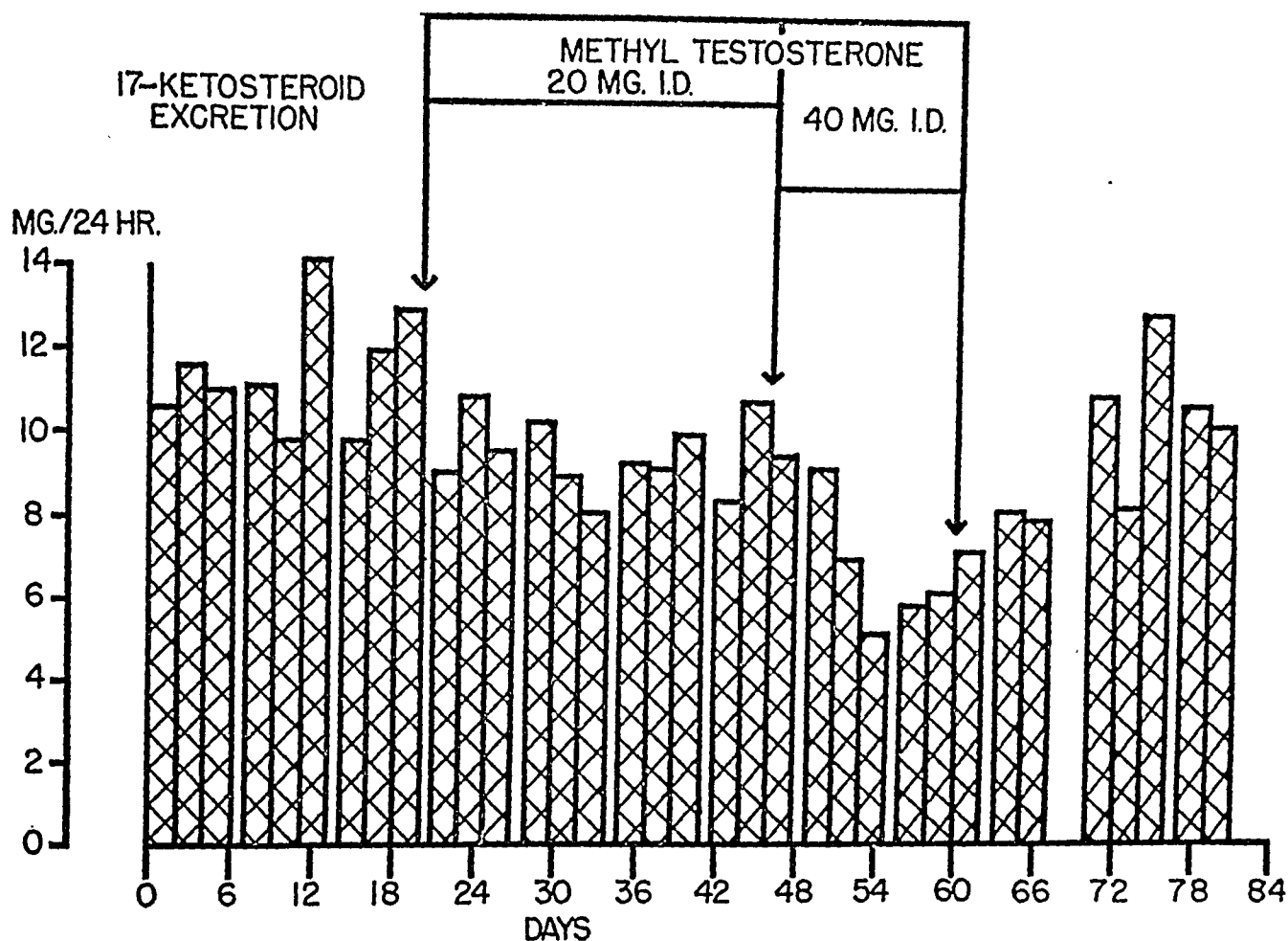


FIG. 9. EFFECT OF METHYL TESTOSTERONE THERAPY ON THE URINARY 17-KETOSTEROID EXCRETION IN CASE 5, A NORMAL WOMAN, AGE 34 YEARS

For discussion, see text. Note that the measurements were made on 24-hour collections.

tration of methyl testosterone, the values for the first 6 days following a change in therapy were omitted in compiling the mean values. In the 9 experiments, the 7 patients had an average excretion of 18.7 mgm. of steroid per 24 hours before, 11.9 mgm. during, and 18.4 mgm. after methyl testosterone administration.

DISCUSSION

The possibility that the decrease in the urinary 17-ketosteroid level during the administration of methyl testosterone is fortuitous and unrelated to the therapy is almost eliminated by the following points: (a) there is a direct relationship between the fall in level and the administration of the therapy, which is clear-cut in Case 1 (both experiments), Case 2, Case 3, Case 5, and Case 7 (Experiment 2); (b) there is a tendency toward a fall in the excretion in *all* of the other experi-

ments (including the studies of the two males with Addison's disease, and of the male with normal function of the adrenals and of the testes); (c) the fall in the level is gradual suggesting a physiologic alteration rather than a chance variation; (d) there is a rebound to higher or pre-treatment levels with cessation of therapy in all of the experiments in which these studies were made except Experiment 1, Case 7; and (e) the amount of decrease (*circa* 40 to 60 per cent) in excretion in Case 1 (both experiments), Case 2, Case 3, Case 5, and Case 7 (Experiment 2) significantly exceeds both the technical errors of the method (*vide supra*) and the amount of unexplained day to day variation (not more than 25 per cent and usually less (20)) that we have encountered elsewhere. For example, 17 assays on one male individual gave an average excretion of 12.9 mgm. per 24 hours with a deviation of ± 1.5 mgm. or

± 12.6 per cent per 24 hours (21). In view of these considerations, it is concluded that the decrease in the urinary 17-ketosteroid level during the administration of methyl testosterone is due to the action of this compound.

Much of the argument to follow depends on the assumption that the 17-ketosteroid excretion in the urine can be used as an index but not a measure of "N" hormone production. We have elsewhere (9) reviewed the considerable circumstantial evidence that the urinary 17-ketosteroids are: (a) largely derived from "N" hormones, and (b) only slightly, if at all, from "S" hormones. We will cite here only one piece of evidence for each of these suppositions. Thus, on the one hand, testosterone (a strong "N" hormone) is excreted largely as androsterone and etiocholanolone both of which are 17-ketosteroids; on the other hand, pre-pubertal children, who in all probability have a normal "S" hormone production, excrete negligible amounts of 17-ketosteroids in the urine.

The experiments herein reported indicate that methyl testosterone interferes with the adrenal cortical mechanism for producing urinary 17-ketosteroids or their precursors. Such an interference might be produced in at least two ways; (a) a direct inhibition by methyl testosterone of

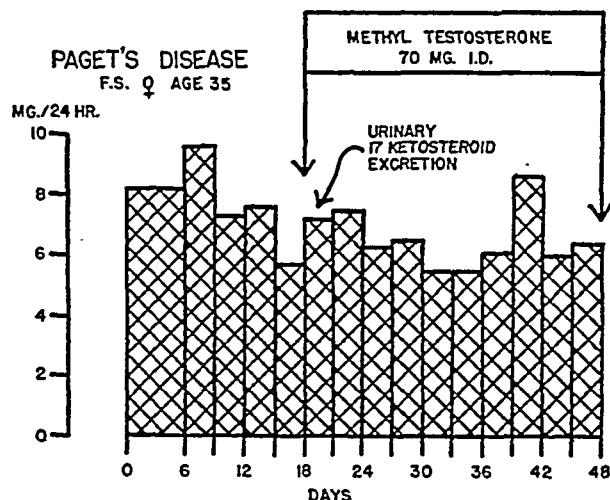


FIG. 10. EFFECT OF METHYL TESTOSTERONE THERAPY ON THE URINARY 17-KETOSTEROID EXCRETION IN CASE 6, A WOMAN WITH PAGET'S DISEASE OF BONE (OSTEITIS DEFORMANS)

For discussion, see text. Note that the measurements were made on a 144-hour collection for days 1 to 6, and on 72-hour collections thereafter.

the adrenal cells which produce 17-ketosteroids or their precursors, or (b) an indirect inhibition of these cells by decreasing the production of some tropic hormone (or hormones) by the anterior pituitary. Animal experimentation favors the latter instead of the former thesis, since it has been shown (22) that the atrophy of the adrenal cortex induced by testosterone therapy does not occur in pituitarectomized animals receiving adrenal corticotrophic hormone. This is analogous to experiments of others (6) in which the damaging effects of testosterone on the Leydig cells could not be produced in pituitarectomized animals in which the Leydig cells were maintained by chorionic gonadotropin (a hormone very similar, if not the same, as the luteinizing hormone).

In the case of the male gonad, the tropic hormone in question is the luteinizing hormone, LH. Since it has been here shown that methyl testosterone inhibits the 17-ketosteroid production by the adrenal cortex and since the same is thought to be true for the male gonad, these experiments may add one more piece of evidence to that obtained elsewhere (*vide infra*) that the production of 17-ketosteroids or their precursors from both these organs is stimulated by the same tropic hormone, namely LH.

TABLE I
Effect of methyl testosterone on urinary
17-ketosteroid excretion

Case No.	Average excretion		
	Before	During *	After *
	mgm. per 24 hours		
Case 1, Exp. 1	15.3	6.1	13.0
Case 1, Exp. 2	13.5	7.6	
Case 2	18.7†	5.9	16.3
Case 3	15.8	11.8	13.2
Case 4	42.8	34.5	40.1
Case 5	11.5	6.1‡	10.2
Case 6	7.6	6.4	
Case 7, Exp. 1	24.2	18.8§	18.0
Case 7, Exp. 2		10.0§	17.7
Average	18.7	11.9	18.4

* Values during first 6 days after change in therapy omitted.

† Control level before any treatment.

‡ Average during administration of 40 mgm. per day.

§ Values during chorionic gonadotropin therapy included in calculating the average excretion (see Figure 11).

|| Experiment 2 immediately followed the after-period of Experiment 1 (see Figure 11).

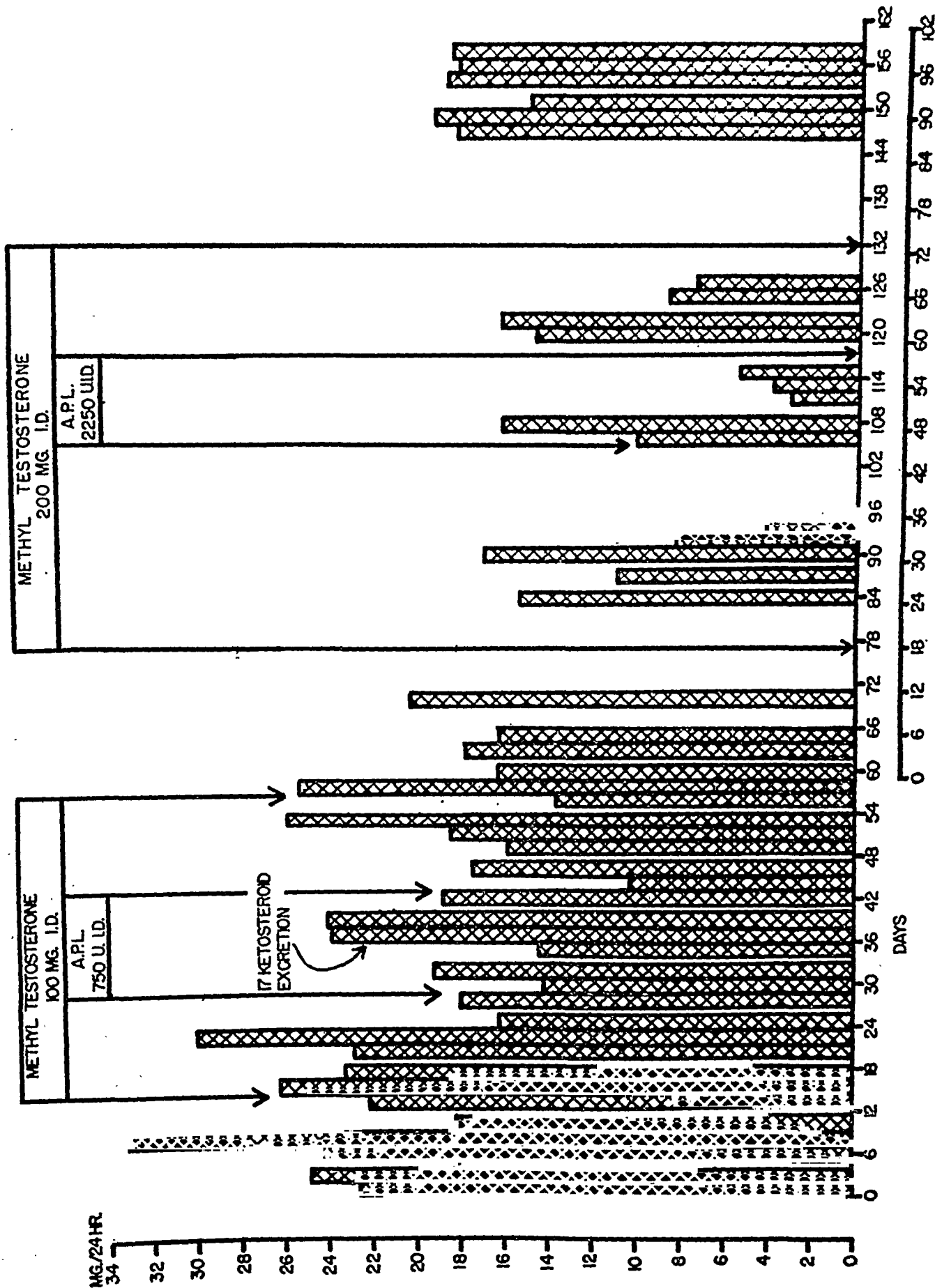


FIG. 11. EFFECT OF METHYL TESTOSTERONE THERAPY ON THE URINARY 17-KETOSTEROID EXCRETION IN CASE 7, AGE 27 YEARS, WITH RUDIMENTARY TESTES. For discussion, see text. Note that the measurements were made on 24-hour collections.

In Figure 12, an attempt is made to show these hormonal relationships schematically in a normal individual; in Figure 13, attempts are made to show these relationships both before and after the administration of methyl testosterone in a normal individual, a female patient with Cushing's syndrome, a female patient with adrenogenital syndrome, and a male patient with Addison's disease.

There is evidence, besides that here presented, that one tropic hormone of the pituitary stimulates in the male both the cells of the adrenal cortices concerned with the production of 17-ketosteroids and the cells of Leydig. In a previous paper from this clinic (14), it was pointed out that those eunuchoid patients (the minority) with high titers of follicle-stimulating-hormone in the urine had 17-ketosteroid excretions of the order of magnitude of those seen in normal females. The inference was that these patients suffered from a primary underfunction of the gonads (*i.e.*, eunuchs from an endocrine point of view) with normal function of the adrenal cortices. It was further shown that those eunuchoid patients (the majority) who did not have a high titer of follicle-stimulating-hormone in the urine had 17-ketosteroid excretions considerably lower than those in the normal female; the inference was that such patients were suffering from a defective production of 17-ketosteroids in both the adrenal cortices and the testes. This suggested that one hormone stimulated both organs (see Figure 12). Furthermore, the failure of bilateral orchidectomy in patients with metastatic cancer of the prostate to lower the 17-ketosteroid excretion (24) is in accord with the suggestion that such a procedure removes an inhibitor to the tropic hormone in question and that the resulting increased production of said tropic hormone stimulates increased production of 17-ketosteroids in the adrenal cortices.

Similarly, the observation that the 17-ketosteroid excretion of young adult females tends to rise for a year or more following termination of ovarian function by castration or x-radiation (25, 26) favors the same interpretation. The demonstration that there is a rise in the urinary excretion of luteinizing hormone in the menopausal state (27) also is in accord with the suggested hypothesis.

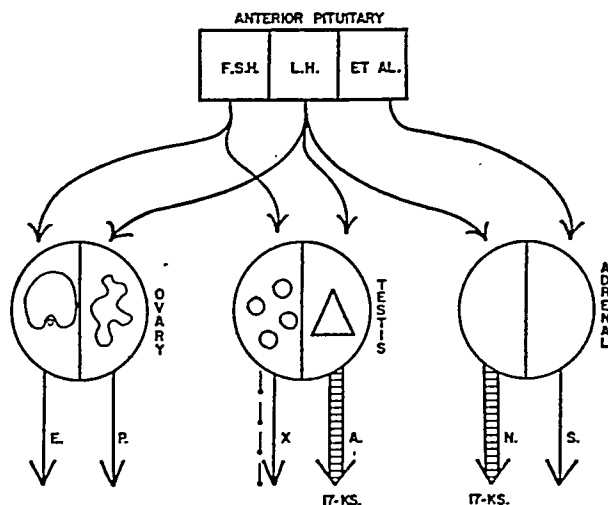


FIG. 12. SCHEMATIC DIAGRAM TO SHOW NORMAL RELATIONSHIP BETWEEN ANTERIOR PITUITARY TROPIC HORMONES AND TISSUES PRODUCING 17-KETOSTEROID PRECURSORS

The pituitary is divided into three compartments: FSH for follicle-stimulating-hormone, LH for luteinizing hormone, and "et al." for remaining hormones. The authors are not certain whether the compartment marked LH should not also include luteotrophin. Striped arrows are used to represent 17-ketosteroid hormones. E—estradiol; P—progesterone; X—hypothetical hormone produced by tubules of testes (23); A—androgen (testosterone); N—"N hormone" (see text); S—"S hormone" (see text).

Other circumstantial evidence that the luteinizing hormone stimulates 17-ketosteroid production by the adrenal cortex is summarized in the following paragraphs. Further discussion of the findings that support these contentions, as well as of a small amount of data that do not, may be found elsewhere (28, 29).

Atrophy of the adrenal cortex has been produced in animals by the administration of testosterone (22, 30 to 40) and progesterone (22, 38, 41, 42); while hypertrophy of the cortex has resulted from treatment with estrogens (43 to 59). These effects cannot be produced in hypophysectomized animals in which the adrenal cortex is maintained in a normal state by the administration of adrenocorticotrophic hormone (22, 45, 52, 60). This is evidence that the effects of testosterone, progesterone, and estrogens on the adrenal cortex are brought about through the pituitary. It has been shown that testosterone (6, 38, 61, 62) and progesterone (63 to 66) inhibit the production of luteinizing hormone (LH) by the anterior

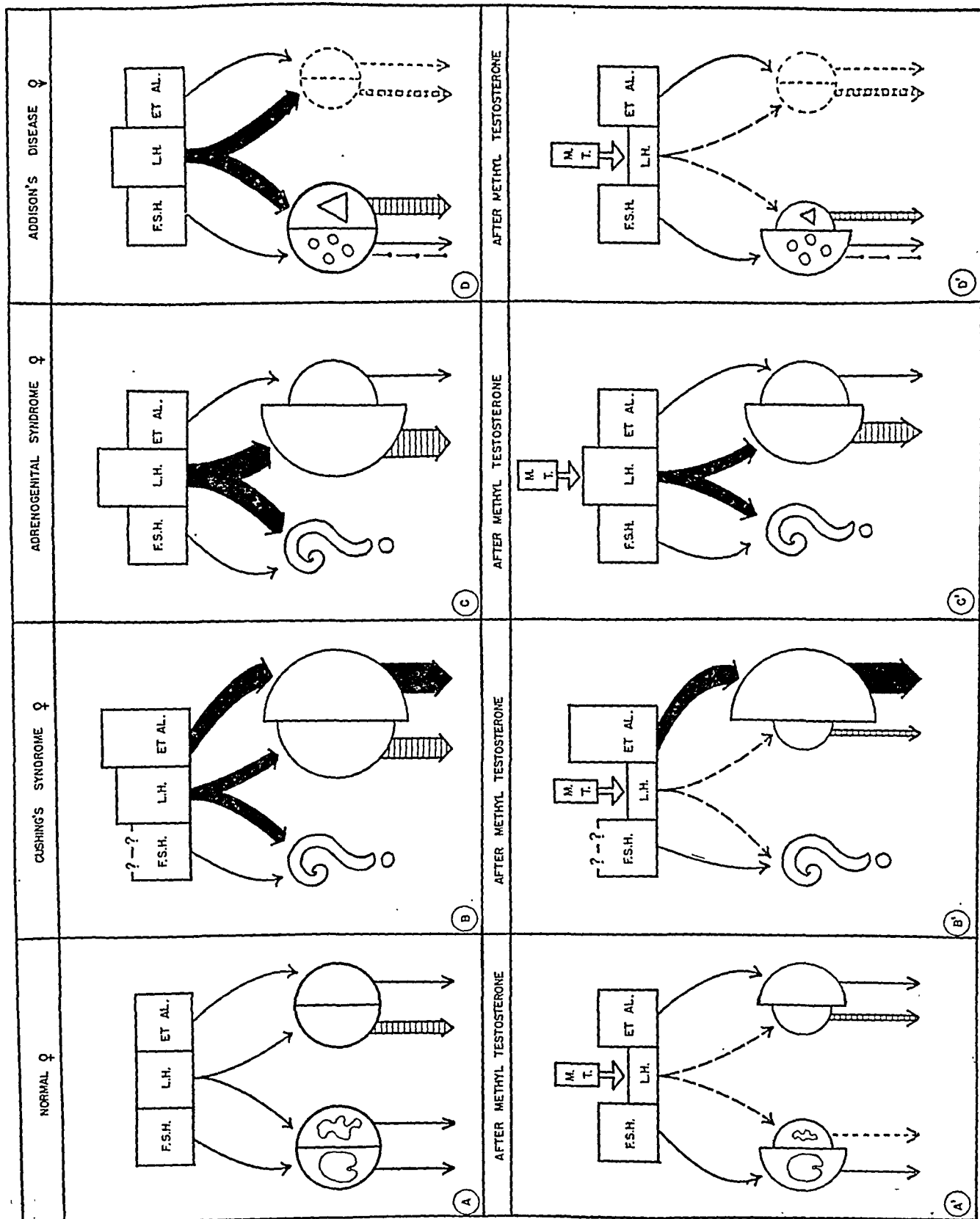


FIG. 13. SCHEMATIC DIAGRAM TO SHOW THE EFFECT OF METHYL TESTOSTERONE THERAPY ON THE RELATIONSHIP BETWEEN ANTERIOR PITUITARY TROPIC HORMONES AND TISSUES PRODUCING 17-KETOSTEROIDS OR THEIR PRECURSORS

The relationships in a normal woman (A) have been described in Figure 12. In the normal woman, after methyl testosterone (A'), there is decreased production of LH, and, therefore, of 17-ketosteroid precursors from the adrenal cortex; ovulation and corpus luteum formation also may be prevented. In Cushing's syndrome (B), corticotrophic hormone ("et al.") and the "S" hormone are shown much increased; LH and 17-ketosteroid precursors are shown moderately increased; in this syndrome, after methyl testosterone (B'), there is decreased production of LH, and hence, of 17-ketosteroid precursors from the adrenal cortex. In the adrenogenital syndrome (C), LH and the 17-ketosteroid precursors are represented as much increased; after methyl testosterone therapy (C'), both LH and the 17-ketosteroid precursors are reduced but not to normal levels. In a male with Addison's disease (D), the adrenal gland is destroyed by disease and its hormones are lacking (indicated by dotted lines); as a consequence there may be a moderate increase in LH, and in turn in the 17-ketosteroid precursors produced by the Leydig cells of the testis. In a male with Addison's disease, after methyl testosterone therapy (D'), LH and therefore the 17-ketosteroid precursors from the Leydig cells are decreased.

pituitary, while estrogens (45, 67 to 69) increase the production of LH. Hypertrophy of the adrenal cortex has been produced by the administration of LH (70), or by substances very analogous to it, such as chorionic gonadotropin (70 to 75) and the gonadotropic principle of pregnant mares' serum (70, 76); presumably atrophy of the adrenal cortex may result from the lack of LH. Further evidence is obtained from the effects of removal of the gonads. Gonadectomy results in an increase in the gonadotropic activity of the pituitary gland (77, 78). Gonadectomy also causes hypertrophy of the adrenal cortex (12, 13, 22, 50, 79 to 88). This hypertrophy of the adrenal cortex can be made to atrophy by testosterone (22, 34, 78, 83, 87, 89, 90) and by progesterone (22, 87); and to increase by estrogens (58, 88). These facts make very attractive the assumption that LH exerts an effect on the adrenal cortex which is characterized by an hypertrophy of the cortex when LH production is excessive, and by an atrophy of the cortex when LH production is diminished below normal.

The question then arises whether the alterations in the adrenal cortex that are attributed to changes in the production of LH by the pituitary cannot be attributed equally well to changes in the production of adrenocorticotrophic hormone (ACTH) of the pituitary, since ACTH also exerts an effect on the adrenal cortex which is characterized by a hypertrophy of the cortex when ACTH production is excessive (50, 80, 91 to 105) and by an atrophy of the cortex when ACTH production is diminished below normal (106 to 116). Against this assumption are the following facts: (1) distinct differences can be demonstrated histologically between the hypertrophy of the adrenal cortex produced by the two mechanisms, since in hypophysectomized animals no restoration of the lipid content and no disappearance of the sudanophobe zone occurs after the administration of LH (52, 76) and estrogens (52, 76), while a disappearance of the sudanophobe zone and restoration of the lipid content occurs after the administration of ACTH (52, 76, 101); and (2) hypertrophy of the adrenal cortex has been produced by LH preparations that contain insignificant amounts of ACTH (52, 70, 76), and *vice versa* (99 to 105). These facts favor the assump-

tion that both LH and ACTH affect the adrenal cortex, but not in the same manner.

The strength of the evidence derived from the animal experiments cited above would be increased if it could be shown that alterations in the size of the adrenal cortex were correlated with changes in the excretion of 17-ketosteroid or androgenic substances. In most of these investigations, this evidence has not been sought. Pertinent to this discussion, however, are the observations of certain investigators (117, 118) who were able to produce significant enlargement of the seminal vesicles and prostates of castrated rats by means of pituitary extracts which also caused hypertrophy of the adrenal cortices. Since the effect could not be elicited in the absence of the adrenals, it is clear that this gland had been stimulated to secrete an androgenic principle. Another author (119) obtained similar results in castrated male guinea pigs, and also observed a masculinizing effect (hypertrophy of the clitoris) with pituitary extracts in ovariectomized females. Studies to demonstrate directly in man the effect of chorionic gonadotropin on the production of 17-ketosteroids by the adrenal cortex are being conducted.

Since the luteinizing hormone in the female stimulates the production of progesterone (120), it might be anticipated that large doses of progesterone (like methyl testosterone) would inhibit 17-ketosteroid production from both the adrenal cortices and the male gonads; studies to answer this question also are being conducted.

The evidence here presented suggests that the high 17-ketosteroid excretion in the adrenogenital syndrome is less easily influenced by methyl testosterone than is that in Cushing's syndrome, even though the pathology in both instances is hyperplasia of the adrenal cortices. This finding is consistent with the thought, already expressed elsewhere (9), that the increased 17-ketosteroid production in Cushing's syndrome is compensatory to the primary pathology which is an increased production of the "S" hormone; such being the case, one would anticipate that when an exogenous source of "N" hormone was made available in the form of methyl testosterone, the endogenous production would quickly recede. In the adrenogenital syndrome, on the other hand, the primary cause of pathology is thought to be a

hyperplasia of those cells which produce "N" hormone, and one would anticipate that it would be harder to modify a process which is primary than one which is secondary.

From the clinical point of view, however, the important point is that the over-production of "N" hormone in the adrenogenital syndrome can be reduced. This may be a clue to therapy. Needless to say it is of little value to such a patient to have her endogenous "N" hormone production decreased by taking an exogenous source of "N" hormone. However, if a steroid could be found which inhibits the endogenous source without itself being an androgen, an important advance in the therapy of these unfortunate patients would probably be at hand. Progesterone has not had a beneficial effect in moderate doses (5, 121); it is to be hoped that it may have in very large doses. In any case, the authors feel very strongly that the approach to the problem is in this direction, as nothing is to be gained by subtotal resection of the adrenal cortices (122, 123).

SUMMARY AND CONCLUSIONS

1. Since 17-methyl testosterone is not excreted as a 17-ketosteroid, it was employed to study the effect of a testosterone compound on the endogenous production of urinary 17-ketosteroids of adrenal origin.

2. The administration of methyl testosterone decreased the urinary 17-ketosteroids of 7 patients in whom the only source of these substances was the adrenal cortices. The patients included: 2 women with adrenal hyperplasia and Cushing's syndrome, 2 women with adrenal hyperplasia and adrenogenital syndrome, 2 women with normal cortical function, and 1 man with adrenal hyperplasia and congenital absence of functioning testicular tissue.

3. The administration of methyl testosterone caused a suggestive but not conclusive decrease in the urinary 17-ketosteroids of 2 male patients with Addison's disease in whom the only source of these substances was the testes.

4. Since the production of 17-ketosteroids or their precursors by both the adrenal cortex and the male gonad appears to be inhibited by methyl testosterone, it is suggested that the mechanism of inhibition is the same for both glands.

5. This inhibition is attributed to a decreased production of some pituitary tropic hormone, as suggested by animal experiments in the literature. If one accepts this hypothesis, the findings here reported support the thesis that the same tropic hormone, presumably the luteinizing hormone, stimulates both glands.

6. The 17-ketosteroid excretion by patients with the adrenogenital syndrome was less easily influenced by methyl testosterone than was that of patients with Cushing's syndrome. This is further evidence that the elevated 17-ketosteroid excretion in the former condition is a manifestation of the primary pathology, whereas the increased excretion in the latter condition is an indication of a compensatory process.

7. The fact that the 17-ketosteroid excretion in the adrenogenital syndrome can be reduced with methyl testosterone shows that the condition is potentially reversible and gives hope that a non-androgenic steroid may be found which will likewise reduce the 17-ketosteroid excretion in this condition.

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TRAUMATIC SHOCK. X. THE TREATMENT OF HEMORRHAGE SHOCK IRREVERSIBLE TO REPLACEMENT OF BLOOD VOLUME DEFICIENCY¹

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Since loss of blood volume is the initiating factor common to most types of traumatic shock, the therapeutic problem has been studied experimentally primarily from this point of view. Confusing or complicating factors, such as local trauma and the sepsis of trauma and anesthetics including barbiturates, have been eliminated by limiting the experimental method to simple hemorrhage in the unanesthetized animal, with or without morphine. Shock, so induced and maintained, exhibits all the classical phenomena expected and is responsive within certain variable limits of time and levels of blood pressure, to the restoration of all the shed blood. If the shock state is treated effectively first by a replacement of the blood volume deficiency, the shock is said to be "reversible" and as such does not represent a problem of challenging import. The problem, for our purpose, begins when and if the replacement of all shed blood, by whole blood or a blood substitute, fails to produce a sustained improvement equivalent to virtual recovery. This communication is concerned with the therapeutic value of a variety of agents administered after the shock has been shown to be irreversible to the infusion of all shed blood. This we regard as the most valid technique for testing the efficacy of any agent.²

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

² The existence of a state of irreversibility to transfusion can be only roughly estimated (see below) because age, condition of the experimental animal, nutritional and fluid reserve, environmental temperature, position on the table, etc. influence the progress of the shock state. Since no reliable method for confidently predicting the response to blood or blood substitute alone has been developed, claims made for the therapeutic value of any procedure or any substance other than blood or a blood substitute could be valid if effective alone and without restoring blood

METHOD

Unselected mongrel dogs weighing from 8 to 20 kgm. were given a single dose of morphine sulphate (1 to 2 mgm. per kgm. intramuscularly) 1 to 2 hours before the start of the experimental period. Groin vessels were exposed after procaine infiltration with a minimum of dissection and blood loss and without aseptic precautions. Sulfanilamide powder or propamidine cream was put in the wound at the end of the dissection and again just before closure. No other wound was created, except for an occasional exposure of the left external jugular vein, through which a tube or catheter was passed to the right auricle for sampling the mixed venous blood or measuring the venous pressure. The arterial blood pressure was measured by a mercury manometer attached to a heparinized cannula in the femoral artery. The animals were handled gently and were lightly tied to the table. The position of the thorax was changed frequently during the shock period and after therapy. In a prolonged course of therapy, occasional animals were removed from the table now and then and allowed to walk about.

The animals were bled rapidly from the femoral artery into clean but unsterilized vessels containing heparin. Since unanesthetized dogs may tolerate blood pressure levels as low as 50 mm. Hg for long periods (in occasional instances for as long as 5 to 8 hours) without developing irreversible shock, bleeding was continued until the blood pressure dropped to 30 mm. Hg and maintained within a range of 30 to 50 mm. Hg by subsequent small withdrawals or reinfusions of blood. The amount of bleeding required to accomplish this fall varied from 20 to 60 ml. per kgm. Subsequent spontaneous drops in blood pressure below 30 mm. Hg were apt to result in cessation of respiration and death, even though shock irreversible to transfusion might not have supervened. These were treated by the infusion of enough blood to raise the blood pressure to just above 30 mm. Hg and to maintain spontaneous respiration. When, after a period varying from 1 to 5 hours, there was little or no response of blood pressure to small test transfusions of 25 to 50 ml. of blood, irreversibility to transfusion was likely, though

volume deficiency or if effective after a transfusion has failed. If the estimate of an agent's value is based upon its presumed effectiveness when given just before or along with the blood or blood substitute, unequivocal evidence must be provided to show that the latter alone would not have achieved the same result.

not certainly predictable. When the dog was thought to be irreversible to transfusion the remainder of the withdrawn blood was filtered through gauze and rapidly infused into the femoral vein. The blood pressure response was then followed. If the condition of an animal was so poor at the end of the shock period that it did not respond to transfusion with a rise in blood pressure to 80 to 100 mm. Hg or higher, it was not used. If the animal recovered completely or maintained a blood pressure level above 80 mm. Hg for more than 3 to 4 hours, it was discarded. Only when the blood pressure fell to 70 mm. Hg or less was irreversibility to transfusion assumed and a test of supplementary therapy started. (The only deviation from this procedure occurred in the group of dogs (Group F) which were treated by sodium bicarbonate dissolved in the transfused blood.) In some experiments, therapy was continued until the animals were clearly moribund or dead; in others, the groin wounds were closed upon termination of therapy and the animals were taken down from the tables. The thoracic and abdominal viscera were examined as soon as possible after death.

In about half of the experiments herein reported, it was found convenient to use an apparatus (1) which is arranged to permit bleeding freely into a bottle suspended at a level corresponding to 30 mm. Hg pressure, so that a rise in arterial pressure above 30 mm. Hg results in further bleeding into the bottle, while a fall below 30 mm. Hg results in back-flow from the bottle into the artery. This steady level of blood pressure is maintained for hours. Sulfanilamide (0.8 gram per 100 ml. of shed blood) and just enough heparin to prevent coagulation were added to the blood in the bottle. With this set-up, irreversibility was considered likely when one third to one half of the blood in the bottle had returned to the dog. The remaining blood was then rapidly infused into the femoral vein. The automaticity of this apparatus made it possible to run as many as 6 experiments simultaneously, so that a group of dogs could be observed under identical environmental conditions.

Since dogs in shock are susceptible to further damage from almost any manipulation, no procedures other than those described were carried out, and blood sampling was almost entirely avoided.

TECHNIQUES OF THERAPY AND RESULTS

Group A: Physiologic saline solution

Five dogs were given 0.85 gram per cent sodium chloride solution intravenously, continuously or intermittently at whatever rate was required to maintain the blood pressure above 80 mm. Hg. If the response was adequate, the rate of injection was controlled so as to minimize overloading of the circulation. If the response was not sustained, the infusion was continued without interruption until the moment of death. The total volume of saline solution given ranged from 75

to 885 ml. per kgm. Transitory improvement of the circulation resulted, as indicated by the rise in arterial blood pressure and the oxygen content of venous blood, but all dogs died within 18 hours after the start of this supplemental therapy. Marked generalized and pulmonary edema, serous effusions, and intestinal hemorrhages were found at post-mortem examination. In many instances, the liver was stiff and swollen, the leaves of mesentery and the lobules of pancreas were separated, and the subcutaneous tissues were tense with fluid.

Group B: Ringer's solution with glucose and alkali³

This preparation was used instead of saline solution in this group to counter a possible deficiency in carbohydrate and the increase in acid metabolites. Six dogs so treated received the fluid after the manner described for Group A, with the same transitory improvement produced by saline solution. All 6 dogs died within 16 hours after the start of this therapy and showed the same autopsy findings as in Group A.

Group C: 5 per cent crystallized bovine albumin⁴

Thirteen dogs received this therapy continuously until they maintained improvement or until they were moribund. The total volume given ranged from 80 to 250 ml. per kgm. In 8 experiments, the albumin was dissolved in 0.85 per cent saline solution; in 5, the albumin was dissolved in the modified Ringer's solution with added glucose and alkali. Temporary improvement in arterial blood pressure and venous blood oxygenation resulted in almost all dogs. Ten died within 24 hours of the start of treatment. One dog, receiving albumin in saline solution, lived 48 hours and one dog, receiving albumin in Ringer's solution, recovered completely.

Marked oozing of blood from all wounds appeared soon after the beginning of the albumin infusion. It could not be controlled by pressure, wound suture, or thrombin solution.

At autopsy, all animals of the group showed

³ Composition of this fluid per liter distilled water: NaCl 8.5 grams, NaHCO₃ 4.0 grams, KCl 0.4 gram, CaCl₂ 0.2 gram, Glucose 50.0 grams.

⁴ Supplied by Armour & Co. through the courtesy of Dr. E. J. Cohn of the Harvard Medical School, Boston.

Therapy of "irreversible" hemorrhagic shock

Nature of therapy	Dogs treated	Survivors	Remarks
A. 0.85 per cent sodium chloride solution 75 to 885 ml. per kgm.	5	0	Rise in venous blood oxygenation with rise in blood pressure. Apparent prolongation of life, but all dead within 18 hours of start of therapy. Generalized and pulmonary edema, serous effusions, intestinal hemorrhages.
B. Ringer's solution with glucose and alkali * 80 to 200 ml. per kgm.	6	0	Looked better while being treated than did dogs in Group A. None survived longer than 16 hours after start of therapy. Autopsy findings as in Group A. Less pulmonary edema. Livers looked better.
C. 5 per cent crystallized bovine albumin 80 to 250 ml. per kgm. Dissolved in saline Dissolved in Ringer's solution with glucose and alkali *	8 5	0 1	Rise in blood pressure and venous blood oxygen content. Apparent prolongation of life, but 7 died within 24 hours. Pulmonary, hepatic, and intestinal congestion and edema. Bloody serous effusion. Hemorrhagic intestinal mucosa. Subserosal and subepicardial hemorrhage. Generalized bleeding (not controlled in wounds by thrombin).
D. 25 per cent bovine albumin in saline 20 to 89 ml. per kgm.	10	0	Small rise in blood pressure. Little apparent effect on course. Dry tissues and serous surfaces. No intestinal hemorrhages (4 dogs).
Physiologic saline given subsequently 50 to 150 ml. per kgm.	6	0	Addition of saline led to some improvement in blood pressure. Development of wet tissues after added saline. Hemorrhage from wounds with rise in blood pressure. All dogs dead within 18 hours after start of therapy.
E. Succinic acid (neutralized) 1 gram per kgm. in saline	13	0	Occasional small rises in blood pressure. No improvement in venous blood oxygen content. No suggestion that survival time was prolonged.
F. Sodium bicarbonate 1 gram per kgm. dissolved in transfusion blood	7	0	Acidosis corrected but poor response to transfusion. All dead within 4 hours. No striking pathology.
G. Pitressin 0.4 to 4.0 pressor units given fractionally plus Ergotamine 0.2 to 0.4 mgm.	13 10	0 0	(All dogs previously treated in Group E.) Small and brief rises in blood pressure. No improvement in venous blood oxygen content. No significant prolongation of survival. Frequent convulsions. Marked blanching and contraction of intestines seen at autopsy. All died within 12 hours.
H. Pitressin 0.5 to 1.0 pressor units in 5 per cent bovine albumin 50 to 80 ml. per kgm.	5	0	All dead within 10 hours after start of therapy.
I. Paredrine 20 to 40 mgm.	6	0	Rise in venous pressure with rise in arterial. No improvement in cardiac output. Duration of effect limited to about 40 minutes.
J. Coramine fractionally to 0.25 gram	4	0	Rise in skeletal muscle tonus to rigidity and convulsions. Inconstant rise in auricular pressure. No improvement in venous blood oxygen content. No rise in arterial blood pressure. No clinical evidence of improvement (All dogs previously treated in Group I).
K. Tuamine 3 mgm. per kgm.	6	0	Increase in arterial pressure but no rise in cardiac output. Duration of effect limited to 10 to 20 minutes and subsequent injections ineffective. No characteristic gross pathology.
L. Potassium phosphate solution 1 to 3 ml. given intracisternally	4	0	Elevation in blood pressure without prolongation of survival time.

* Composition of Ringer's solution with glucose and alkali, per liter distilled water: NaCl 8.5 grams, NaHCO₃ 4.0 grams, KCl 0.4 gram, CaCl₂ 0.2 gram, MgCl₂ 0.2 gram, Glucose 50.0 grams.

marked venous congestion of lungs, liver, kidney, effusions in the pleural, pericardial, and peritoneal mesentery, and gastro-intestinal tract, and large sacs. Many of these effusions were grossly

bloody and there were frequent sub-pleural, sub-epicardial, and sub-peritoneal hemorrhages. Although the tissues were wet, the extreme edema of saline treated dogs was not seen.

Group D: 25 per cent bovine albumin in saline

Ten dogs were given 20 to 89 ml. per kgm. of a 25 per cent solution of crystallized bovine albumin in saline solution. The resulting rise in blood pressure was slight and there was little apparent effect on the shock state.

Four dogs died without further treatment. At autopsy, the tissues and serous surfaces were dry and there was no hemorrhage in the intestine or elsewhere.

In the remaining 6 dogs, when it was clear that no benefit had resulted from the albumin solution, supplementary physiologic saline solution was given intravenously, in doses ranging from 50 to 150 ml. per kgm. Some improvement in blood pressure resulted, but all dogs were dead within 18 hours of the start of therapy. These dogs showed wet tissues at autopsy. With improvement in blood pressure, whether resulting from albumin solution alone or following the supplementary saline, marked bleeding from wounds occurred.

Group E (See Group G below): Succinic acid

Thirteen dogs were given sodium succinate (1 gram per kgm.) in 0.85 per cent saline solution, intravenously. Occasional small rises in arterial blood pressure followed these injections, but no rise in venous blood oxygen content was observed. When the results of therapy were clearly disappointing, further therapy, described under Group G, was given. All 13 dogs died without evidence that the survival time was prolonged.

Group F: Sodium bicarbonate

Seven dogs were given sodium bicarbonate (1 gram per kgm.) dissolved in the shed blood before reinfusion. In several instances, mild hemolysis due to the salt was noted. The blood CO₂ combining capacity showed that acidosis was corrected and did not return. The response to transfusion plus sodium bicarbonate was poor and all dogs were dead within 4 hours after transfusion. There was no remarkable feature in the gross pathology of the viscera.

This is the only group in which the therapeutic agent was given before the response to transfusion alone had been observed.

Group G (See Group E): Pitressin and ergotamine

Thirteen dogs which had previously been unsuccessfully treated with succinic acid (Group E) were given repeated injections of pitressin, the total dose ranging from 0.4 to 4.0 pressor units. The injections were followed by small and brief rises in blood pressure without improvement in venous blood oxygenation. Ten of these dogs also received 0.2 to 4.0 mgm. of ergotamine. Generalized convulsions were seen frequently. There was no evidence of prolongation of survival time. All 13 dogs were dead within 12 hours after the start of therapy. Marked blanching and contraction of the intestine were seen at autopsy.

Group H: Pitressin in 5 per cent bovine albumin

Five dogs were each given 0.5 to 1.0 pressor unit of pitressin, dissolved in 50 to 80 ml. per kgm. of 5 per cent bovine albumin. No significant improvement was noted and all dogs were dead within 10 hours of the start of therapy.

Group I (See Group J): Paredrine (p-hydroxy-a-methylphenylethylamine hydrobromide)

Six dogs were given paredrine in repeated injections. The total dose was 20 to 40 mgm. Rises in arterial and venous blood pressure resulted but without improvement in cardiac output. The pressor effect lasted some 40 minutes and subsequent re-injection produced no response. All 6 dogs died several hours thereafter.

Group J (See Group I): Coramine (pyridine-beta-carboxylic acid diethylamide)

Four dogs which had become unresponsive to paredrine (Group I) were given repeated injections of coramine, reaching a total dose of 0.25 gram. An increase in skeletal muscle tone occurred, culminating in generalized rigidity and convulsions following the last injection. The doses of coramine just short of producing convulsions produced inconstant rises in right auricular pressure, no improvement in venous blood

oxygen content, no rise in arterial blood pressure, or any other evidence of improvement.

*Group K: Tuamine (2-aminophetane sulfate)*⁵

Six dogs were treated after transfusion with tuamine given intravenously in a dose of 3 mgm. per kgm. Of these, 2 were given the drug at a time when the blood pressure was still about 90 mm. Hg, that is, before irreversibility had been demonstrated by relapse into shock. The remaining 4 were treated in the usual fashion, after the increased blood pressure had again fallen to 70 mm. Hg. All 6 dogs died. The observed effects of the drug were an increase in arterial blood pressure, in pulse rate, and in rate and depth of respiration; but no rise in cardiac output was observed. The duration of effect was limited to 10 to 20 minutes and subsequent injections produced no response. The gross post-mortem findings did not differ from those of irreversible hemorrhagic shock treated by transfusion alone.

*Group L: "Potassium phosphate" given intra-cisternally*⁶

Four dogs were given "potassium phosphate" by intra-cisternal injection when shock had recurred after transfusion. An injection of 1 ml. resulted in a marked rise in arterial blood pressure accompanied by nystagmus, proptosis, and stiffening of the neck and back muscles. Subsequent injections to 3 ml. produced no further rise in blood pressure and were followed by cessation of respiration. Doses of 0.3 ml., however, given repeatedly elicited responses in blood pressure over a period of 1 to 2 hours. The effect on the cardiac output was not measured. More extensive investigation was prevented by the small amount of material available. All dogs in the group died and there was no evidence that the survival time was prolonged by the treatment. Post-mortem examination of the spinal cord and brain was not done.

DISCUSSION

Earlier work from this laboratory (2, 3, 4) has

⁵ We are indebted to Dr. Irvine H. Page for this material.

⁶ We are indebted to Dr. Baird Hastings for this material which he obtained on a recent visit to Russia where it was said to have been beneficial in the therapy of shock. Its exact composition is unknown.

demonstrated that the failure of transfusion to restore the animal in hemorrhagic shock is not due to loss of the restored fluid through abnormally permeable capillaries. It has long been clear that restoration of normal blood volume alone is incapable of reversing the deteriorating course of advanced hemorrhagic shock. The experiments herein reported, in which relatively enormous volumes of 5 per cent bovine albumin were infused after the initial transfusion, show that even sufficiently sustained increases in blood volume (to twice or three times normal) do not cure hemorrhagic shock which is irreversible to an initial blood transfusion.

Others (5, 6) have considered the problem from a simple mechanistic view and presented data to the effect that the deteriorating trend in shock in any stage can be halted or even reversed by the simple expedient of intravenous physiological saline solution, provided it is given in sufficient volume and for a sufficient length of time with care to avoid fatal pulmonary edema. One author explained the survival of his animals as due to the creation of a sufficiently high interstitial pressure to reverse the flow of fluids from out to into the circulatory bed. This postulate cannot apply to the type of shock which exists in the presence of an adequate blood volume and is therefore applicable only in the special circumstances of his experiments. The therapeutic value of the saline solution is not confirmed by our data on hemorrhagic shock even though the volume of saline solution was adequate to achieve the tissue pressures demanded by such a postulate. Furthermore, the futility of infusion of relatively huge volumes of fluids which escape only slowly from the circulation (albumin solution) makes it quite clear that when there is no continuing local loss of fluid into a damaged area, the shock state, even though initiated by a loss of blood or plasma, results in the development of changes that are no longer reversible by restoration and maintenance of a normal or more than normal blood volume.

Certain workers (7) burned dogs by immersion in hot water. Death resulted in a few hours if no treatment was given and survival was only slightly prolonged by treatment with saline solution. Longer survival was achieved by saline and soda bicarbonate solution. If plasma was added to this

latter therapy, no substantial benefit was conferred. But if defibrinated blood was added, recovery from the immediately lethal effects resulted until the late secondary effects of tissue damage set in. They explain these results as follows: Saline solution alone may be expected to be useless since the plasma loss is not compensated for. The advantage of adding soda bicarbonate lies in its correction of the acidosis and in the supplying of additional sodium ion. Plasma given with chloride-bicarbonate solution is not effective because, they believe, the plasma leaks out too rapidly, not only into the burned area but into other tissues, so that not only is blood volume not sustained, but edema of the brain and lungs "due to plasma" will produce lethal effects. Since red cells do not leave the blood stream, defibrinated blood maintains blood volume while the chloride-bicarbonate solution restores normal interstitial fluid and electrolyte balance.

To show that plasma is inferior to blood in these circumstances would require the use of larger quantities than were given. The assumption that plasma is futile because plasma leaves the circulation very rapidly when the plasma volume is normal does not imply that it will leave as rapidly when the plasma volume is deficient. There is no evidence to show that large quantities of plasma *per se* produce edema of the lungs or brain. When such edema has been observed, it has occurred only when saline has been given along with it, and the saline is admittedly a more obvious cause of such edema. In numerous experiments on the treatment of hemorrhagic shock, plasma alone was not observed to produce edema until the prelethal phase of shock had arrived and then only when saline solution was added. In these circumstances, edema and bleeding into tissues were observed also if whole blood was used instead of plasma.

In any event, conclusions drawn from the treatment of the acute hemodynamic imbalance of burns by these agents are not valid for simple hemorrhagic shock, since our therapy for the latter condition included the use of whole blood plus saline and soda bicarbonate (plus other normal electrolytes) with no appreciable benefit. Moreover, the infusion of large amounts of albumin or blood with saline or Ringer's solution containing

alkali leads to uncontrollable hemorrhage in open wounds and in the intestine.

Since the restoration of normal circulation is a prerequisite to recovery of tissue function, one may take as early evidence of recovery from shock—aside from restoration of normal blood volume—a rise in cardiac output, an acceleration of capillary flow, a rise in oxygen content of the mixed venous blood, and a rise in blood pressure. Such changes are readily achieved during the early shock phase while it is still reversible to transfusion. Such changes also are achieved readily in the late phase of shock by the intravenous infusion of almost any compatible fluid. But these changes do not persist after the infusion has stopped or even while the infusion is continued. Since this is the case even if the blood volume replacement is adequate, the critical deficiency must be elsewhere within or outside the circulatory apparatus.

Evidence has been provided (8) of declining myocardial efficiency in the late shock phase. Recently, another worker (9) identified by ballistographic observations on the exposed heart a measure of cardiac weakness which was not reversible by any known methods of therapy except certain aliphatic amines ("one-amine," "tuamine," etc.) with specific myocardial stimulating qualities. In 6 experiments with our technique of determining irreversibility, tuamine produced no rise in cardiac output or other beneficial response except a transient rise in systolic pressure. The effect of tuamine was analogous to that of paredrine. Our experiments differed from those mentioned above in that there the drug was given with or immediately after transfusion, whereas we withheld the drug until the response to transfusion alone could be determined.

The fault in the circulation in shock has been attributed (10) to a loss of skeletal muscle tone with consequent failure of venous pressure and return flow. Paredrine constricts arteries and veins and raises pressure in both (11). But improved blood flow does not result, as cardiac output measurements (11) and capillary microscopy of omental vessels⁷ indicate. The evidence does not contra-

⁷ Unpublished data.

dict the above postulate, which can only be tested by an agent which maintains unrestricted capillary inflow while increasing venous pressure. Coramine increases intramuscular pressure (12). In our experiments with coramine, skeletal muscle tone was increased to the point of extreme rigidity without improving blood flow. A more desirable physiological technique to test the hypothesis would provide for alternating contraction and relaxation of muscle.

Some authors (13) believe that the primary deficiency lies in the collapse of the contractile power (vasomotion) of arterioles and venules, which in late shock lose their reactivity to adrenalin. While no specific agent capable of restoring vasomotion has become available, the favorable response reported by them (14) from the use of pitressin with and without ergotamine was not substantiated by our experiments. They gave these agents with the first transfusion as well as subsequently. As already stated, we do not regard this method of testing the efficiency of any agent on the course of the shock state as valid unless it is given without transfusion or after the transfusion is shown to be ineffective.

While the critical deficiency in late shock may lie in an altered physiology of the circulation independent of controlling factors from outside of the circulation, it is natural that such controlling factors should also come under scrutiny, since the effect of inadequate blood flow is all-embracing. Ample evidence of serious disturbance of kidney (15), liver (16, 17), and intestinal (18) function and of extensive biochemical abnormalities, especially in intermediary carbohydrate metabolism (19), is available. Certain investigators (15) have succeeded in defining the limits of anoxia which the kidney can endure before irreparable damage to function results. But the effects of loss of kidney function, while capable of causing death within a few days, are not, as far as is known, operative within the shock period. Collapse of liver function might well result in irretrievable effects and death within the shock period. This problem is now under intensive investigation. We have found in work to be published from this Laboratory (17) that the maintenance of the circulation of the liver of the dog in shock

by cross circulation with a healthy donor animal, prevents the development of "irreversible" hemorrhagic shock under experimental circumstances similar to those described in this report.

Uncompensated acidosis has long been known to persist in the untreated late shock phase and the alkali therapy introduced (20) during the first World War has most recently been recommended by others (21), who attribute to sodium bicarbonate the capacity to reverse and to cure a degree of shock not amenable to transfusion alone. They accept as a test for such a degree of shock not the fact of a futile transfusion already given, but a CO_2 combining capacity of 15 volumes per cent or less. Such a test, if reliable, would be of great value. Our experience is that irreversibility to transfusion can exist with CO_2 values higher than 15 volumes per cent. Indeed, in most experiments, we found it difficult to keep the animal alive long enough to achieve a level as low as 15 volumes per cent. Whether our use of morphine accounts for this difficulty remains to be determined. However, if care was taken to correct acidosis with the initial transfusion and to maintain a normal or higher than normal CO_2 combining capacity thereafter, the deteriorating trend was not noticeably alleviated.

The anoxia of shock, as anticipated, has been found to alter the normal sequence of reactions in carbohydrate metabolism. Phosphorylation of co-enzymes (cocarboxylase and cozymase) essential in carbohydrate breakdown and resynthesis is seriously affected (22, 23). The lack of substrates normally available for energy release has led to efforts to replace them by others present in tissues but not necessarily serving this purpose, e.g., succinic acid. When some workers (24) showed that this substance *in vitro* increases oxygen consumption of tissue slices at low oxygen tensions, others (25, 26) reported substantial improvement in the shock state as a result of its administration. We were unable to substantiate these claims in tourniquet shock (27) or in hemorrhagic shock (Group E). Recently, it has been found (28, 29) that the increased oxygen consumption at low oxygen tensions is produced by succinic acid at the expense of the tissue and to that extent succinic acid can be regarded as deleterious rather than helpful.

The tolerance of the organism to hemorrhage, when the latter is induced by the withdrawal of blood by simple needle puncture of a large artery in the intact unanesthetized and unimmobilized dog, contrasts sharply with that of the dog anesthetized, immobilized, cannulated, and otherwise traumatized. This points up the need of excluding as far as possible all extraneous factors, if the shock due to hemorrhage alone is to be properly evaluated. The more such factors are eliminated, the more difficult it becomes to produce irreversibility to transfusion, which is achieved by prolonging the time and increasing the degree of hypotension. Our experiments approached the ideal of simplification only very approximately, for we used morphine and open wounds and a considerable degree of immobilization in order to obtain essential data not otherwise possible. The factor of sepsis is, however, of dubious import, because of the absence of pathological evidence of its presence in these experiments, and because the experimental period seems too short for the production of significant amounts of bacterial toxin. Even if bacteremia is common in these circumstances, it remains to be shown that the kind of organisms and their number and their virulence were of sufficient consequence during the period of experimentation to have influenced the results. Reduced to the simplest conditions it is possible to achieve experimentally, we believe that the course of events in hemorrhagic shock leading to the development of a state of irreversibility to transfusion is a function of the severity and duration of inadequate capillary flow, with a cumulative adverse effect on the integrity of function of one or more vital organs, such as the liver, or of a basic biochemical process dependent on cellular integrity over a wider area.

The almost immediate precipitation of typical traumatic shock by a potent toxin (30) without a significant fall in blood volume represents a condition analogous to that of the animal in hemorrhagic shock which is irreversible to transfusion. Shock due to such a toxin may be regarded merely as a variant of hemorrhagic shock in the sense that the disruption of cellular integrity is effected immediately by a toxin rather than gradually by the progressive effects of anoxic damage.

Objections have been made to our employing as

a test of irreversibility the failure of the transfusion of all shed blood to reverse the course of the shock process, because, when such a transfusion is given, some dogs will have deteriorated to a greater degree than others; that is to say, that some will be so to speak "10 per cent dead" and others "90 per cent dead" when the blood is returned. Certainly a more convenient test of irreversibility would be desirable, but no reliable one is available. We could wish for more uniformity in the degree of deterioration than we achieve, but this would be possible only if we had some reliable physiologic or biochemical measurement to detect the onset of irreversibility. A certain limitation to the degree of deterioration is obtained by testing the therapeutic agent only in such dogs as show a good response to transfusion before relapsing into shock. A permanently sustained response to transfusion constitutes a therapeutic problem which is solved. What we seek is the understanding of and a method of treating that phase of shock which requires something more than blood alone to correct it. This is the therapeutic problem which is unsolved. Even though the organism irreversible to a transfusion is properly characterized as "10 per cent dead" or "90 per cent dead," it is shock nevertheless and it is the kind of shock that constitutes the existing challenge in therapeutics. To use a less rigid criterion of irreversibility is to accept a less severe test and one, therefore, which is not as safe for testing the effectiveness of any agent aside from the transfused blood itself.

SUMMARY AND CONCLUSIONS

The therapeutic value of various agents for the treatment of hemorrhagic shock, which is not responsive to the replacement of all shed blood, was tested under conditions calculated to avoid or to minimize the confusing effects of anesthesia, blood sampling, operative manipulations, and other forms of trauma. Utilizing the relatively simplified set of conditions described the following results were observed with the agents tested:

1. Massive infusions of saline may cause transitory improvement in circulation but do not cure hemorrhagic shock irreversible to transfusion.

2. Massive infusions of isotonic bovine albumin greatly increase the blood volume and may sustain

the circulation for a time, but only rarely result in recovery. A marked bleeding tendency is produced by this therapy. Concentrated (25 per cent) bovine albumin solution in equivalent or greater protein content is of no benefit, even if supplemented by saline solution.

3. Large volume intravenous infusion therapy, using either saline solution alone or albumin in saline solution, is harmful by producing marked edema of tissues, serous effusion, venous distention, and widespread hemorrhage from small vessels.

4. Pitressin, with or without ergotamine, is of no value. The combination of pitressin with 5 per cent albumin solution is not beneficial.

5. Paredrine causes an elevation of the arterial and venous blood pressure and no improvement in cardiac output. The duration of this effect is limited by the rapid development of unresponsiveness to the drug and survival time is not prolonged.

6. Coramine increases skeletal muscle tone, but does not favorably influence the course of events.

7. The correction of acidosis by the administration of sodium bicarbonate with the initial transfusion does not alter the deteriorating trend of advanced hemorrhagic shock.

8. Sodium succinate is of no benefit in the therapy of advanced hemorrhagic shock.

9. Tuamine, given when the initial transfusion is failing, causes a transitory rise in blood pressure but the effect is brief. Survival time is not prolonged.

10. "Potassium phosphate" intra-cisternally did not alter the deteriorating trend of hemorrhagic shock and at the same time produced undesirable cerebral excitatory phenomena.

It is our view that advanced shock constitutes a state of progressive deterioration which is not amenable to the types of therapy now available, probably because fundamental biochemical changes have developed as a result of prolonged deficiency of capillary flow. These changes may result from injury predominantly involving one vital organ, such as the liver, or from widespread cellular damage.

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TABLE I
Absorptive capacity of jejunal loops in normal dogs

Postoperative time *	Dog No. 30	Dog No. 31	Dog No. 33
<i>hours</i>	<i>per cent</i>		
5 per cent glucose in distilled water			
1.0	60		56
1.75 to 2.25	42	45	50
3.0 to 3.50	37	45	49
4.0 to 4.33	40		48
0.85 per cent saline solution			
1.0	17.5		33
1.75 to 2.50	0	19	27
3.0 to 3.50	14	13	26
4.0 to 4.33	14		27
Water			
1.0	33		60
1.75 to 2.50	20	67	56
3.0 to 3.50	20	54	43
4.0 to 4.33	40		60

* Time after preparing loops. Each determination was made upon a freshly washed loop refilled with the same volume of test material.

capacity is markedly impaired (Table II). Of 19 dogs in whom several glucose absorption determinations were obtained, 18 showed a substantial and progressive decrease in glucose absorption as shock deepened and as the blood pressure continued to fall. The nineteenth dog, which was among 4 dogs whose blood pressure level was above 60 mm. Hg for a substantial period prior to transfusion, did not show a decrease. Determinations of glucose absorption after transfusion were obtained in 15 dogs of the series. Of these, 4 showed some improvement in absorptive capacity and a fifth, which also received bovine albumin, showed marked improvement. Although the blood pressure response was satisfactory in all of these, improvement did not occur in others in which this response was equally satisfactory. The absorptive capacity after transfusion generally declined from the initial post-transfusion value, whether the blood pressure remained up or declined.

Absorption of water. Water absorption from a jejunal loop likewise varies considerably among unshocked dogs recovering from ether anesthesia given for the preparation of loops (Table III). The disparity among such dogs is not so great for the absorption of water as of glucose. Of 13 dogs in which serial water absorption determinations were made during hemorrhagic shock, all showed

a marked decrease in absorption as shock deepened. Four showed improvement in water absorption following blood transfusion and a fifth showed marked improvement following an infusion of 5 per cent bovine albumin. The post-transfusion capacity for water absorption was sustained whether the blood pressure did or did not decline subsequently. This is in contrast to the continuing decline in the absorptive capacity for glucose after transfusion.

Absorption of physiologic saline solution. One will note the marked difference in the absorptive capacity for physiologic saline solution between unshocked dogs emerging from ether anesthesia and unshocked dogs who had local anesthesia only (Table IV). While there are occasional instances of decline in the absorptive capacity during shock, there is in general no clearly defined effect on this function for physiologic saline. Indeed, contrary to the figures for water and glucose, not a few instances of improved absorption are noted after shock has deepened, only to decline again considerably as shock continues to deepen after an ineffective transfusion.

DISCUSSION

The volume flow of blood through the capillary circulation of the intestine in shock is less than normal (3) and the rate of flow is also below normal, if flow through the mesentery or the omentum may be taken as a gauge (4). This circumstance presumably explains the decline in the rate of absorption of intraluminal fluids and electrolytes. The absorption of glucose, however, is not a simple diffusion process across a semi-permeable membrane (5). Phosphorylation must precede its absorption. That phosphorylation is disturbed in shock because of anoxia has been shown (6 to 8) in various organ systems. Since deficient blood flow and anoxia coexist in all types of shock, and since all organs are involved, including the pancreas and liver which may be unable to deliver their normal complement of digestive juices and bile, it is not improbable that the absorption of almost any normally absorbable food and fluid elements is likely to be deficient.

The absorptive capacity of the small intestine in severely bled dogs has been measured (9) and similar findings noted except for glucose, the ab-

TABLE II
Rate of intestinal absorption of glucose

NORMAL			SHOCK			AFTER THERAPY		
Dog No.	B.P.	Absorption	Duration	B.P.	Absorption	Interval	B.P.	Absorption
	mm. Hg	per cent	hours	mm. Hg	per cent	hours	mm. Hg	per cent
1*	120	38.3	2.0	30	14.4			
2*	110	20.6	4.0	50	6.5			
3*	110	32.2	3.0	65	25.2			
4*	120	56.0	3.0	55	45.0	2.25	80	26.6
5*	125	76.0	2.0 3.5	55 40	60.0 44.0	1.50	100	42.0
7*	90	87.5	1.5 3.5	70 55	37.5 27.7	1.50	90	19.9
8*	95	84.7	2.0	30	24.5	2.5	115	39.7
9*	100	53.3	2.5	30	6.5			
10*	85	19.9	2.0 3.75	50 65	19.5 4.5	0.5 1.75 alb. i.v. 1.0	85 75 90	8.7 8.7 26.6
11*	100	19.2	2.25 3.25 4.0	45 50 30	5.2 4.6 0	0.5 1.5	90 70	5.2 1.2
12*	80	20.6	1.0 1.75 2.5	65 60 45	22.8 12.5 20.6	0.75 1.5	45 30	15.3 10.5
13*	100 95	47.8 42.6	1.0 2.0 2.5	60 75 40	43.0 31.8 14.8	1.5 2.25 3.25 2nd trans. 1.0 2.0	85 90 70 90 90	18.7 12.4 22.6 8.1 15.0
14*	110 115	20.6 15.5	1.0 2.25 3.0	75 65 60	23.8 44.3 48.6	1.5 2.0	130 110	22.0 19.6
16*	90	14.9	0.75 2.5	50 35	13.1 6.6	2.0	40	6.6‡
17*	120 110	6.5 6.5	1.75	45	0	0.75 2.5	110 30	0 6.5
19*	105 105	33.2 28.0	1.5	70	16.3	1.75 3.75	105 85	27.1 21.9
20†	130 120	30.8 26.7	1.5	20	14.0	1.0 2.5	95 50	20.0 11.0
21†	130 140	41.5 39.0	1.0 1.75	70 50	26.5 12.1	1.25 2.25	120 120	28.3 6.5
32†	100 100	60.9 57.1	0.75 1.5	45 40	36.4 14.0	0.75 1.5	100 100	41.2 35.0

* Ether anesthesia.

† Local anesthesia.

‡ Saline and pitressin i.v. after blood.

TABLE III
Rate of intestinal absorption of water

NORMAL			SHOCK			AFTER THERAPY		
Dog No.	B.P.	Absorption	Duration	B.P.	Absorption	Interval	B.P.	Absorption
	mm. Hg	per cent	hours	mm. Hg	per cent	hours	mm. Hg	per cent
8*	95	77.7	2.0	30	28.6	2.5	115	50.0
9*	100	83.4	2.5	30	33.3			
10*	85	50.0	2.0	50	17.9	0.5	85	14.3
			3.75	65	14.3	1.75	75	21.4
						alb. i.v. 1.0	90	53.5
11*	100	87.0	2.25	45	26.8	0.5	90	0
			3.25	50	36.8	1.5	70	0
			4.0	30	20.0			
12*	80	63.5	1.0	65	63.7	0.75	45	66.7
			1.75	60	46.7	1.50	30	53.5
			2.50	45	33.3			
13*	100	100	1.0	60	85.8	1.50	85	28.5
	95	100	2.0	75	50.0	2.25	90	64.2
			2.5	40	14.2	3.25	70	71.4
						2nd trans. 1.0	90	56.2
						2.0	90	56.2
14*	110	86.5	1.0	75	60.0	1.5	130	8.6
	115	76.5	2.25	65	7.9	2.0	110	10.0
			3.0	60	7.9			
16*	90	100	0.25	50	33.2	2.0	40	0†
			2.50	35	6.7			
17*	120	90.0	0.75	70	43.3	0.75	110	23.3
	110	66.6	1.75	45	33.3	2.50	30	26.5
18*	100	75.0	1.0	60	50.0	1.0	110	25.0
	100	55.0	2.0	40	25.0			
19*	105	66.0	1.5	70	0	1.75	95	6.7
	105	33.0				3.75	50	6.7
20†	130	80.0	1.5	20	30.0	1.0	95	0
	120	55.0				2.5	50	5.0
21†	130	80.0	1.0	70	10.0	1.25	120	10.0
	140	50.0	1.75	50	10.0	2.25	120	0
32†	100	92.6	0.75	45	57.4	0.75	100	40
	100	92.6	1.50	40	20.0	1.50	100	40

* Ether anesthesia.

† Local anesthesia.

‡ Saline and pitressin i.v. after blood.

sorption of which was found uninfluenced by the hemorrhage. These experiments are not strictly comparable to ours, since no effort was made to produce a sustained state of shock; in addition, only one posthemorrhage determination was made while we made successive absorption tests as shock continued. The relative ease of absorption of physiologic saline after hemorrhage was first noted

by these workers and we are able to confirm this finding. The explanation for this finding may lie in the greater need for physiologic saline in shock than for water or isotonic glucose and in the small amount of work and therefore lower peripheral circulatory efficiency required for its absorption than for the absorption of water or glucose solution.

TABLE IV
Rate of intestinal absorption of saline

NORMAL			SHOCK			AFTER THERAPY		
Dog No.	B.P.	Absorption	Duration	B.P.	Absorption	Interval	B.P.	Absorption
	mm. Hg	per cent	hours	mm. Hg	per cent	hours	mm. Hg	per cent
8*	95	70.0	2.0	30	32.1	2.5	115	39.7
10*	85	14.7	2.0	50	20.3	0.5 1.75 alb. i.v. 1.0	85 75 90	9.1 7.6 11.9
11*	100	0	2.25 3.25 4.0	45 50 30	22.8 17.1 10.6	0.5 1.5	90 70	20.3 8.9
12*	80	6.5	1.0 1.75 2.5	65 60 45	18.7 25.9 27.6	0.75 1.5	45 30	2.4 10.6
13*	100 95	15.9 8.8	1.0 2.0 2.5	60 75 40	14.2 0 0	1.5 2.25 3.25 2nd trans. 1.0 2.0	85 90 70 90 90	0 0 0 10.7 7.9
14*	110 115	0 11.7	1.0 2.25 3.0	75 65 60	20.1 17.6 3.4	1.5 2.0	130 110	5.9 65.6
16*	90	45.0	0.75 2.50	50 35	43.0 9.2	2.0	40	0†
17*	120 110	0 4.6	0.75 1.75	70 45	14.2 15.9	0.75 2.50	110 30	15.9 9.2
18*	100 100	17.0 0	1.0 2.0	60 40	6.8 0			
19*	105 105	0 1.9	1.5	70	13.1	1.75 3.75	105 85	9.8 1.9
20†	130 120	25.0 25.0	1.5	20	17.0	1.0 2.5	95 50	8.0 8.0
21†	130 140	40.0 47.5	1.0 1.75	70 50	35.5 25.6	1.25 2.25	120 120	6.1 0
22†	120 120	27.9 43.0	0.75 1.50	50 30	30.8 0	1.0 2.5	105 110	4.8 33.3
23†	120 120	50.7 19.0	0.75 1.50	70 40	23.6 61.3	1.0 3.0	140 120	34.6 47.8
24†	150 150	15.4 1.7	0.75	65	18.8			
26†	140 140	37.7 32.4	0.75 1.50	45 35	32.4 42.0	0.75 1.50	90 70	7.5 3.7
27†	130 120	32.4 30.0	0.50 1.50 2.25	60 45 45	20.3 31.6 26.4	0.75 2.0	105 105	20.7 7.9
29A†	120 115	51.0 56.0	0.75 2.25	70 45	43.5 33.2	0.50 1.50	110 110	5.9 29.9
29B†	120 115	18.8 35.2	0.75 2.25	70 45	28.8 21.2	0.5 1.5	110 110	29.8 39.0
29C†	120 115	37.0 37.8	0.75 2.25	70 45	35.2 17.2	0.5 1.5	110 110	9.4 14.1

CONCLUSIONS

In hemorrhagic shock, the small intestine is progressively deficient in its absorptive capacity for water and isotonic glucose. After transfusion, whether effective or not, some improvement in this function may be observed, but full recovery is not observed within the time interval of these experiments.

The absorption of physiologic saline is not clearly affected by the institution of hemorrhagic shock until the advanced stage of shock is reached.

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COLD AGGLUTININS. I. OCCURRENCE OF COLD ISOHEMAGGLUTININS IN VARIOUS CONDITIONS

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The development of significant titers of cold agglutinins (autohemagglutinins) in most cases of primary atypical pneumonia of unknown etiology¹ (1, 2) was first noted in this laboratory (3) and later, independently elsewhere (4 to 6). This finding has since been confirmed in other clinics (7, 8). A few cases of pneumonia are included among the previously reported cases of marked autohemagglutination collected from the literature in 1937 (9) and a detailed study of some of the important properties of the cold agglutinin was carried out in a case of "diffuse bronchopneumonia" reported in 1918 (10). Cold agglutinins have also been described in many other conditions, notably in association with various blood dyscrasias, liver diseases, and in allergic and peripheral vascular disorders. Their occurrence even in these conditions is still sufficiently rare that only individual cases or very small groups of cases have been reported by any single observer. The only infectious disease in which autoagglutinins have been found with any great regularity is trypanosomiasis (11).

The literature concerning the occurrence of cold agglutinins, their nature and properties, and the methods for detecting them have been reviewed recently (12). Very few reports are available of extensive surveys of the occurrence of cold agglutinins in various infectious diseases with the use of standardized quantitative methods. In a study of sera from 421 cases of various common infectious diseases (6), significant titers were found only in cases of primary atypical pneumonia and of mumps complicated with orchitis. Many of the sera included in that study had been stored for several months. Other authors (7, 8) obtained negative results, in 135 and in 138 control

cases respectively, including normals and persons with various infections other than primary atypical pneumonia.

The present paper deals with the results of tests for cold agglutinins in 1069 cases of a variety of common diseases particularly those involving infections of the respiratory tract. Details concerning the occurrence of cold agglutinins in the 200 characteristic cases of primary atypical pneumonia included among these cases and the results of studies of some of the properties of the cold agglutinins are left for separate consideration in the papers which follow.

MATERIALS AND METHODS

Patients. Except for some of the patients with atypical pneumonia from other hospitals, almost all of the cases studied were regular admissions to the wards of the Boston City Hospital between September 1942 and March 1944. Control sera included a number of serial specimens provided by Drs. T. Duckett Jones and Benedict F. Massell, from carriers and non-carriers of hemolytic streptococci, as well as from 6 cases of rheumatic fever. Many of these had hemolytic streptococcal infections shortly before or during the period when the bloods were being collected. Many cases were chosen specifically for this study as representatives of disease entities, others were selected because they had febrile illnesses of more than a few days duration while still others were included because they were receiving sulfonamide therapy for a week or longer. Almost all of the patients with bacterial pneumonias and about two-thirds of the remaining cases, other than those without disease, received sulfonamide therapy. The diagnosis recorded in each case was arrived at by the authors after reviewing all of the available data.

Both serum and plasma were used in the tests. Venous blood was obtained in most cases under sterile precautions and allowed to clot at room temperature or at 37° C. The clear serum was then separated and removed after centrifugation. Some of the tests were carried out immediately but usually the sera were stored in sterile rubber stoppered test tubes in a refrigerator at 5 to 10° C. and the tests were carried out in most instances within a

¹ Hereafter referred to as "atypical pneumonia."

TABLE I
Cold isoagglutinins in various conditions

Diagnosis	Maximum titer					Total
	Less than 10	10	20	40	80 and over	
Primary atypical pneumonia, etiology unknown	45	8	10	22	115	200
Probable atypical pneumonia		1	5	2	3	11
No disease: includes 33 carriers and 37 non-carriers of hemolytic streptococci	100					100
Pneumonia: Pneumococcal	143	5	1	1	1	151
Staphylococcal	32	2		1	2	37
Streptococcal	18		2			20
Other bacterial	1	1				2
Pneumonia, probably bacterial, etiology not determined	86	2	2			90
Acute respiratory tract infections without pneumonia:						
Tracheobronchitis	30	1				31
Pharyngitis, tonsillitis, sinusitis	59	1				60
Clinical influenza	18					18
Influenza (virus or antibody)	43	1	1		1	46
Tuberculosis: Miliary	8					8
Pulmonary (positive sputum)	12	1	1			14
Pleural effusion (positive guinea pig)	5	2	2		1	10
Others	2					2
Chronic pulmonary infections and tumors	12	1	1	1		15
Sterile pleural effusions (? etiology)	7					7
Bronchial asthma with acute bronchitis	11			1		12
Burns with lung involvement (including 5 with hemoglobinuria)	19					19
Congestive cardiac failure	24					24
Pulmonary (7) and cardiac (9) infarctions	16					16
Enteric and urinary tract infections	26	2				28
Exanthemata: Rubella	5			1		6
Measles	8					8
Varicella	5					5
Mumps (3), Pertussis (1), Diphtheria (1)	5					5
Meningococcal (10), pneumococcal (6), influenzal (1), and streptococcal (2) meningitis	17		2			19
Acute rheumatic fever	16					16
Sulfonamide rash (not otherwise included)	5					5
Erythema multiforme (vesicular type)	13					13
Streptococcus viridans (7) and pneumococcal (4) endocarditis	11					11
Gonococcal infections	4	1	1			6
Misc. surgical infections	19					19
Poliomyelitis (1), lymphogranuloma venereum (2), lymphocytic choriomeningitis (1)	4					4
Diabetes (1), periarteritis nodosa (1), hepatic cirrhosis (5), internal hemorrhage (3), thrombophlebitis (1)	11					11
Fever of undetermined origin	6					6
Thrombocytopenic purpura (2), infectious mononucleosis (3), acute leukemia (2)	7					7
Hemolytic anemias (no pneumonia)	1	1	1		4	7
Totals (excluding primary atypical pneumonia)	809	21	14	5	9	858

few days. A number of specimens were obtained as oxalated blood. These were warmed to 37° C. and the plasma, separated by centrifugation, was used for the tests on the day it was obtained or on the following day. Serial specimens were taken at suitable intervals in most of the cases but single samples were obtained in some of them, usually during the second to the fourth week after onset of the febrile illness.

The definition of cold agglutinins for purposes of this study is the one usually accepted for human bloods. The

agglutination is independent of the blood groups, is optimum at 0 to 5° C., diminishes with rise in temperature, and is no longer demonstrable at 37° C. For convenience, the tests were done for cold isoagglutinins rather than for autoagglutinins which are usually, though not always, entirely comparable. The erythrocytes in these tests were obtained from group "O" donors. A few members of the laboratory staff contributed all of the blood used and most of the cells were obtained from 3 of these donors. Oxalated blood was washed 3 times in a large volume of

saline and a 2 per cent suspension of cells in physiological saline was then made and used in the tests. For the present study, almost all of the tests were carried out with cells that were 2 to 4 days old.

The tests for cold agglutinins were carried out as follows: Serial 2-fold dilutions of serum or plasma in saline were made in 0.5 ml. amounts in 100 × 11 mm. test tubes. In most of the early tests, the first dilution was 1:2 but in the later ones it was 1:5. Equal volumes of 2 per cent cells were added, the tubes were shaken thoroughly, and stored in a refrigerator overnight. The racks of tubes were usually placed in an ice water bath before the readings were made. In reading the tests, the tubes were rapidly inverted 3 times to loosen all of the cells from the bottom of the tube and to allow thorough mixing. The agglutination was graded from 4+ to 1+, the former representing a single hard clump of cells not broken up on mixing while the latter indicated a finely floccular agglutination, easily visible without magnification. Finer agglutination, visible only with the aid of a magnifying lens (\pm), was noted but not considered in the values recorded. The titers are recorded as the reciprocal of the highest final dilution of serum giving 1+ agglutination.

After the readings were made in the cold, the racks of tubes were placed in a water bath or incubator at 37° C. for 2 hours. All the positive agglutinations recorded in these studies were completely reversed by this procedure.

RESULTS

A summary of the maximum titers obtained in the 1069 cases of various diseases is given in Table I. It is seen at a glance that two groups of cases stand out from among all the others. They are the cases of atypical pneumonia and those of hemolytic anemia. Of the 200 characteristic cases of atypical pneumonia, 137 or 68.5 per cent had a maximum titer of 40 or higher. The cases listed as "probable atypical pneumonia" each had a similar characteristic clinical course but either the lung lesion had already cleared when the first specimen of blood was obtained, or the history and physical signs were characteristic, but the x-ray was negative, or complicating factors such as congestive heart failure or superimposed bacterial infections made the interpretation of the pulmonary findings uncertain. All of these cases had cold agglutinins, but a titer of 40 or higher was obtained in only 5 of them.

Among the cases other than those of atypical pneumonia, cold agglutinins were found in the greatest frequency and highest titers in the

hemolytic anemias. Significant titers were obtained in 4 of 7 such cases and low titers (10 or 20) were obtained in 2 of the others. The bloods in all of these cases were studied by Dr. T. Hale Ham in considerable detail. A few of the significant facts in the 4 cases with the high titers may be noted.

The first of these was in a colored man of 35 who was first studied by Dr. Ham in 1939. At that time he had a Lederer's type of acute hemolytic anemia with cold agglutinins in a titer of 160 (1+) and strong (2 or 3+) agglutination in the lower dilutions. The patient had latent syphilis at the time and more than 2 years later he was treated in the hospital for luetic meningitis, but his anemia had cleared by then and he no longer had cold agglutinins.

The second patient was a colored man, 45 years old, who was first admitted on February 12, 1943, and was found at that time to have hypertensive heart disease, syphilitic laryngitis, and an acute upper respiratory tract infection. At this time, the patient may have had atypical pneumonia which was not recognized because of the other findings. No abnormal blood cytological findings were made out during this admission and tests for cold agglutinins were not done. On March 11, 2 weeks after discharge, he was readmitted because of hemoglobinuria and severe anemia. The cold agglutinin titer was 1280 on March 11 and dropped to 320 during the next 6 weeks. The agglutination was marked (3+ and 4+) in all but the highest dilutions. The anemia improved and the patient was discharged but he again returned 2½ months later, at which time he died of congestive heart failure without a recurrence of the anemia. The cold agglutinin titer at this time, however, was 2560 (2+) and again the agglutination was very marked (4+ and 3+) in all but the highest dilution.

The third case was that of a woman, 45 years old, who had long suffered from cold urticaria and hemoglobinuria. In this case, too, the cold agglutinin titer was 2560 with marked agglutination occurring in all but the last dilution of serum.

The fourth case was in a colored man of 34 who developed a marked acute hemolytic anemia on the sixth day of treatment with sulfanilamide for acute glomerular nephritis and streptococcal pharyngitis. Cold agglutinin titers of 40 and 80 were obtained in 2 samples of serum on the day after the anemia and hemolysis occurred and after the sulfanilamide was discontinued. Three weeks later, the titer was still 80. Strong agglutination (3+ and 2+) occurred in the 1:10 and 1:20 dilutions in each instance.

In all of these 4 cases, there was increased osmotic fragility of the red blood cells (only a slight increase was demonstrated on the first day in the last case mentioned), the Donath-Landsteiner and acid hemolysis tests (13) were negative, and the cold agglutination was completely abolished at 37° C.

Among the remaining 851 cases, only 10, or 1.2 per cent, had cold agglutinins in titers of 40 or higher. A few pertinent remarks concerning the illness and the course and character of the cold agglutination observed in these cases are also of interest. There were 5 such cases among those listed as bacterial pneumonias.

One of these cases was in a man 42 years old, who had an abrupt onset of typical lobar pneumonia on March 2, 1943, without antecedent upper respiratory tract infection. He was admitted on the following day and received full doses of sulfapyrazine for 6 days. His temperature fell to normal on the second day, only to return almost to the original level of 104° on the following day and the fever persisted until the tenth day. During this time, there were physical and x-ray signs first of frank consolidation of the left upper lobe and then of atelectasis of that lobe followed by extension of the consolidation to the lower lobe. The leukocyte counts varied from 27,000 to 11,000 during the febrile course. Type 7 pneumococci predominated in cultures of the sputum obtained at entry and repeated blood cultures were negative. A cold agglutinin titer of 40 (1+) was obtained on the seventh day and a slightly stronger agglutination (2+) was noted only in the 1:10 dilution of the same serum. The second case was one of a type 7 pneumococcal pneumonia with negative blood cultures, leukocytosis, and a good response to penicillin. A titer of 160 was obtained both in acute-phase and convalescent sera. The patient had a severe cough for 3 weeks prior to the abrupt onset of the lobar pneumonia.

In the other 3 cases, *staphylococcus aureus* was the only or predominant organism. The first of these was in a woman 22 years old whose illness began on September 22, 1943, and was characterized by chilliness, substernal and right lower chest pain, cough, and purulent sputum. There were physical and x-ray signs of dense consolidation of the left lower lobe and possibly of fluid, but the latter was not demonstrated by thoracentesis. Sputum and blood cultures obtained on the fifth and ninth days were positive for hemolytic *staphylococcus aureus*. The leukocyte count stayed between 8500 and 9400 throughout the febrile course. There was gradual improvement in the fever and symptoms on sulfamerazine therapy. Drug therapy was discontinued after 10 days because of the occurrence of a rash and fever. The cold agglutinin titer was 40 (2+) on the fourteenth day and 80 (2+) on the twenty-first day. The early clinical course in this case strongly suggested that of a primary atypical pneumonia with superimposed staphylococcal infection.

In the second case of staphylococcal pneumonia there were a number of complicating factors. The patient was a woman 56 years old who was admitted on February 10, 1943, with a variety of symptoms which, together with the physical and laboratory findings, were interpreted as those of cirrhosis of the liver and chronic lymphogranuloma venereum. There were scattered medium moist râles, and x-rays showed fine miliary nodular areas of density

throughout both lung fields. The patient had a persistent low grade fever and leukopenia. She also had an active osteomyelitis of a finger and *staphylococcus aureus* septicemia. The pulmonary lesions persisted throughout her stay. She died after 7 weeks in the hospital and autopsy was not obtained. On 2 occasions in the middle of her hospital course, her blood showed cold agglutinins in a titer of 80 (1+) with somewhat stronger agglutination (2+) in all the lower dilutions. In this case, also, the possibility that the pulmonary lesion began as a primary atypical pneumonia cannot be entirely excluded.

The last case among the pneumonias was in a man of 64. He was admitted in October 1943 on the third day of an illness which began with chills, diarrhea, headache, pleuritic pain in the left lower chest, dyspnea, and cough productive of grossly bloody sputum. He had had a slight coryza 3 days prior to the onset of these symptoms. There were physical and x-ray signs of consolidation of the left lower lobe. The leukocyte counts ranged between 3000 and 7100. *Staphylococcus aureus* predominated in cultures of the sputum, and the blood cultures were all negative. On sulfamerazine therapy, the patient's temperature dropped in 2 days from 102.8 to about 100° F. and a low grade fever persisted for another week. In this case, a cold agglutinin titer of 40 (1+) was noted on the sixteenth day but there was no stronger agglutination observed in any of the lower dilutions of the same serum.

One patient with tuberculous pleurisy with effusion had cold agglutinins. He was a man of 40 who had had a slight cough for a year until one week prior to his entry on February 22, 1944, when he began to have chilly sensations, sweats, and substernal pain. Characteristic fluid was obtained which yielded tubercle bacilli on guinea pig inoculation. Fever reaching 102° F. daily persisted throughout his hospital stay, during which he received 2 courses of sulfapyrazine therapy. The cold agglutinin titer before sulfonamide therapy was started was 80 (1+) with somewhat stronger agglutination (2+) in the lower dilutions. After the first course of sulfapyrazine, the titer was 40 (1+). In this case, too, the early symptoms preceding the onset of the effusion suggest the possibility that the patient had atypical pneumonia which activated a pre-existing tuberculosis. Similar reactivation of tuberculosis has been observed in this clinic following definite influenza virus infections. It is of interest that all 4 of the other cases of tuberculous pleurisy with effusion which were studied showed some cold agglutinins in titers of 10 and 20. In each of these cases, however, the agglutination was weak (1+) even in lower dilutions.

One patient with a proved carcinoma of the lung had a cold agglutinin titer of 40. In this case, however, the agglutination was weak (1+) in all the dilutions that were tested. The patient's symptoms consisted of cough, bloody sputum, anorexia, and loss of weight. Signs of fluid predominated in the affected side of the chest and characteristic bloody fluid was removed and shown to contain tumor cells.

The case of rubella was a typical one with a slightly

prolonged febrile course. Cold agglutinins in a titer of 40 (1+) were demonstrated in serum obtained several days after the fever and rash had subsided. The agglutination was somewhat stronger (2+) in the lower dilution of the same serum. There was no clinical evidence of pneumonia in this case except for a slight cough.

The patient with bronchial asthma was a man of 70 who was admitted February 24, 1943, two days after the onset of an acute tracheobronchitis. There were scattered crepitant as well as musical râles throughout the chest and physical and x-ray signs of bronchiectasis and of patchy bronchopneumonia in both lower lobes. He had a febrile course of 11 days in spite of sulfadiazine therapy. Blood taken on the eighth day had a cold agglutinin titer of 40 (1+) and the agglutination was weak (1+) in the lower dilutions as well.

The cases of influenza are of special interest. Of the 64 cases studied, all except 2 of those from which virus was isolated occurred during the course of the epidemic in December 1943 and early in 1944 (14). Serial samples of serum were tested in most of these cases. In 2 cases, there was weak cold agglutination (1+) in titers of 10 or 20 and the same titers were observed in all the sera from each case. In only one case did cold agglutinins develop to a significant titer.

This case was in an intern who had the characteristic symptoms of clinical influenza which began February 26, 1944, several weeks after the peak of the influenza epidemic in Boston. He had been in Minnesota during the previous November and December when the epidemic of influenza was at its height there but he had no symptoms at that time. His clinical course was characterized by daily swings in temperature from normal to 101° F. for 4 days, with cough and headache throughout this time. There were a few scattered fine râles heard only in his right lower lobe for several days but repeated x-rays revealed no abnormal findings in the lungs. The leukocyte counts remained normal throughout his course. A few pneumococci, Type 28, were obtained in cultures of throat washings obtained for virus studies on the day after the onset. These washings were injected intranasally in mice and into the allantoic sac of 11-day chick embryos and readily yielded a virus which had many of the characteristics of an influenza virus but it differed serologically from typical strains of both A and B influenza viruses. This strain is now being studied further. No rise in antibodies to either the PR8 (type A) or the Lee (type B) strains of influenza could be demonstrated by either the complement fixation or the agglutination-inhibition (Hirst) tests and no rise was demonstrated by the latter test against his own virus.² The sera obtained on the second and ninth days showed no cold agglutinins but those of the sixteenth and thirty-second days had titers of

20 (2+) and 80 (2+), respectively. Such titers have been obtained at corresponding stages of the disease in many characteristic but mild cases of primary atypical pneumonia.

From the data presented, it is apparent that among the cases other than those of hemolytic anemia, some at least may have had atypical pneumonia. In them, either positive x-ray signs were not made out or the symptomatology and physical signs were obscured by those of other coexisting diseases. Even in one of the cases of hemolytic anemia,—the one which was complicated by syphilis and hypertensive heart disease,—the appearance of or increase in cold agglutinins may very well have resulted from the acute respiratory infection which occasioned the first entry. This infection, in turn, had many characteristics of atypical pneumonia, but, unfortunately, studies with this in mind were not made until the patient was readmitted later with hemoglobinuria and anemia. In fact, x-rays taken for other purposes during the first admission showed suggestive lesions in the lungs which had been overlooked. The signs of atypical pneumonia, if that is what he had, were completely overshadowed by those of syphilitic laryngitis and congestive failure.

DISCUSSION

The data presented indicate quite clearly that significant titers of cold agglutinins, demonstrable by the method used, are found infrequently in normal individuals and in patients with respiratory or other infectious diseases except in cases of primary atypical pneumonia and in certain types of hemolytic anemia. Indeed, in some of the few other cases in which similar titers of cold agglutinins were demonstrated, the possibility of recent or coexisting atypical pneumonia could not be entirely ruled out. In fact, a detailed review of the other cases in which cold agglutinin titers of 10 or 20 were found in this study showed a similar likelihood of the coexistence of atypical pneumonia in many of them. From the literature it would appear that only in cases of trypanosomiasis have cold agglutinins been encountered with the same regularity.³ Furthermore, a careful study of

² This patient is listed as Case 68 in reference 14.

³ A high incidence of cold agglutinins has been reported recently in cases of tropical eosinophilia (16) and among Melanesian natives in yaws epidemic areas (17).

the previously reported cases of marked auto-hemagglutination suggests that many of them may have had pulmonary infections similar to those which are currently being classified as atypical pneumonia. Favour (15), however, found cold agglutinins more frequently in other conditions.

At this time, it is still reasonable to say that no single specific etiological agent has as yet been identified in cases of primary atypical pneumonia and consequently no reliable immunological test based on such a specific etiological agent is available for the definite identification and classification of such cases. The development of cold agglutinins may, therefore, serve as a helpful diagnostic aid in these cases until such agent or agents are discovered. At least the development of cold agglutinins in patients presenting characteristic symptoms and physical and x-ray signs may serve to segregate one group of cases among those now classified as primary atypical pneumonia of unknown etiology. It also seems reasonable to assume that the development of cold agglutinins in these cases is related in some way to the unknown causative agent. This does not entirely preclude the possibility that other cases having the same etiological agent may run a similar clinical course without developing cold agglutinins and, conversely, that certain other types of etiological agents may produce cold agglutinins. The relation to the disease or to the development of cold agglutinins of the virus isolated from the patient who was considered, on clinical grounds, as having influenza is still obscure.

In this study, the tests for cold agglutinins were carried out in each case within a few days of the time when the blood was drawn. This is an important factor, particularly in sera of low titer as will be shown in a later paper in this series. Deterioration on storage of the cold agglutinins in sera of atypical pneumonias was postulated by Horstmann and Tatlock (7) because of the significantly lower titers found by them in sera that had been stored for a long time before they were tested.

SUMMARY AND CONCLUSIONS

Tests for cold agglutinins, using erythrocytes from normal group O donors, were carried out in 1069 cases, including normal persons and patients

with a variety of conditions, mostly respiratory tract infections of various sorts.

Cold agglutinins in titers of 40 or higher were found in 137 or 68.5 per cent of 200 characteristic cases of primary atypical pneumonia of unknown etiology, in 5 of 11 cases in which this was the probable diagnosis, and in 4 of 7 cases of various kinds of hemolytic anemia.

In only 10, or 1.2 per cent of the remaining 851 cases were cold agglutinins demonstrated in similar titer. In some of these 10 cases and of the few others in which cold agglutinins were demonstrated in lower titers, atypical pneumonia may have played a part.

It is suggested that the development of cold agglutinins is related to the causative agent of primary atypical pneumonia and may serve as a diagnostic aid in this disease.

The authors are deeply indebted to the many physicians, both at the Boston City Hospital and elsewhere, who permitted us to see and follow their cases, obtained the blood samples, and made available the clinical and laboratory data. Dr. T. Hale Ham provided some of the sera and studied the blood in the cases of hemolytic anemia and Miss Geneva A. Daland carried out some of the tests in these cases.

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COLD AGGLUTININS. II. COLD ISOHEMAGGLUTININS IN PRIMARY ATYPICAL PNEUMONIA OF UNKNOWN ETIOLOGY WITH A NOTE ON THE OCCURRENCE OF HEMOLYTIC ANEMIA IN THESE CASES

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In the preceding paper (1), there was presented a summary of the maximum titers of cold agglutinins found in 1069 cases of a variety of conditions, including the various infections of the respiratory tract that are commonly encountered in this vicinity. Significant titers (in dilutions greater than 20) were obtained only in cases of primary atypical pneumonia of unknown etiology,¹ and in a few other cases particularly those of hemolytic anemia. The present paper is concerned solely with an analysis of the results of the tests for cold isohemagglutinins in 200 cases with characteristic clinical and x-ray findings of atypical pneumonia. A preliminary report of some of these findings has already been published (2).

PATIENTS, MATERIALS, AND METHODS

Selection of cases. All of the 200 cases included in this study occurred between September 1942 and January 1944, inclusive. Most of them were from among the regular admissions to the medical wards of the Boston City Hospital during this period, but 45 of them occurred in other hospitals. Almost all of the latter were seen on one or more occasions by one of the authors, and the bloods and the clinical and laboratory data were obtained and generously put at our disposal by their respective physicians. All of the cases fulfilled the criteria for the diagnosis of primary atypical pneumonia of unknown etiology (3, 4), including definite and characteristic x-ray findings of involvement of at least an appreciable portion of one lobe. Doubtful cases in which either the x-ray or clinical findings were not entirely characteristic or in which a primary bacterial or virus etiology was determined or strongly suspected, were excluded for purposes of this study. Fifteen fatal cases are included. Autopsies were obtained in most of these cases and confirmed the clinical diagnosis in each instance.

Methods. The method used in the tests for cold agglutinins was given in detail in the preceding paper (1). For the present study, the specimens of venous blood were all collected under sterile precautions and the serum was separated from the clotted blood, either at room tempera-

ture or, more often, after the blood had been kept at 37° C. for a short while. The cleared sera were stored in rubber stoppered tubes at 5 to 10° C. Specimens, averaging 3 per patient, were obtained at suitable intervals. The tests were carried out either on the day when the blood was obtained or within a few days. Equal volumes of serum and 2 per cent saline suspensions of 2- to 4-day-old red blood cells from individual group O donors were used. The titers are recorded as the reciprocals of the highest final dilutions of serum giving 1+ agglutination at 0 to 5° C. and complete dispersion at 37° C.

RESULTS

The conditions under which the present cases were studied unfortunately did not permit frequent observations throughout the entire course of the acute illness and prolonged observations during convalescence in every instance. Data concerning certain of the features to be considered are, therefore, based on limited numbers of cases. In the analysis which follows, the time relationships are referred to the day of onset of symptoms. In most of the cases, this could be ascertained quite accurately, but, in a few instances, it was only approximated.

Time of appearance, decline, and disappearance

The *first positive tests* (Figure 1A) were observed between the seventh and twenty-seventh days, but mostly during the second or third week. The last negative tests in the same cases were obtained before the end of the second week in all but 2 of them. The *maximum titers* (Figure 2) occurred mostly between the eleventh and twenty-fourth days. There were a number of cases, however, in which the maximum titers were not attained until the fifth week or later, and in 4 cases, maximum titers of 40 to 160 were observed between the fourth and eighth days and lower titers obtained later. There was no definite correlation between the maximum titers and the time

¹ Hereafter referred to for brevity as "atypical pneumonia."

they were attained. The *first significant drop* from the maximum titer occurred mostly between the third and fifth weeks (Figure 1B) and cold agglutinins could no longer be found or were present in titers of 20 or less between the fourth and sixth weeks in most cases (Figure 1C). In 3 cases, however, significant titers were still present after 2 months and disappeared during the next month. Titers in representative cases are shown in Figure 3.

Histories of illustrative cases

Certain relevant features of 2 cases are shown in Figures 4 and 5. These cases were in interns on one of the medical services and one of them may have acquired his disease from the other. A summary of their histories follows.

The illness in *Case 32* (Figure 4) began with malaise, chilly sensations, fever, headache, and a hard cough which increased in severity and was dry at first but later became productive of yellow mucoid sputum. The lungs were clear to physical examination and x-ray at the time of admission. After a week, however, showers of fine râles appeared in the left axillary region and then spread to involve the lower half of that lung. A few râles were also heard in the base of the right lower lobe at the end of the second week. X-rays showed fine mottling in the corresponding areas. Cold agglutinins were first demonstrated in the serum in the middle of the second week, rapidly reached a maximum titer, and then declined appreciably towards the end of the fourth week. Agglutinins for the indifferent streptococcus 344 (5) first appeared at the same time as the cold agglutinins and were present at the end of the fourth week.²

Case 58 (Figure 5) was that of an intern who cared for the previous patient. Sixteen days after his colleague first took sick, he began to have marked fatigue, generalized aches, fever, headache, and soreness of the eyes, followed by cough and a tightness in the chest. Physical examination on admission was negative except for the fever. The patient then had repeated chills and sweats. Showers of medium crepitant râles appeared and spread gradually to involve the entire left lung and later the right lower lobe. X-rays showed diffuse, finely mottled densities in the corresponding areas. Fever lasted 2 weeks and the lungs then began to clear gradually and were entirely clear before the patient was discharged from the hospital. Cold agglutinins first appeared in low titer on the ninth day, reached their peak during the third week, and then dropped rapidly. Agglutinins for streptococcus 344 were found in low titer during the third week. Sulfonamide drugs were not used in this case or in the previous one.

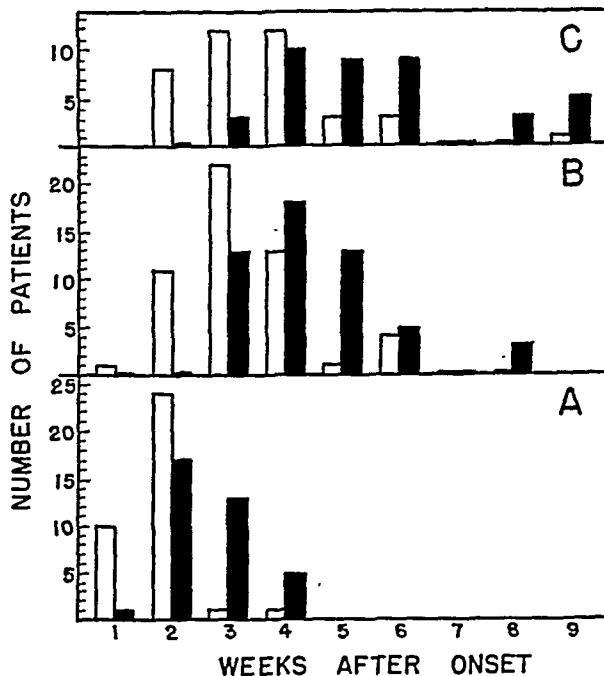


FIG. 1. TIME OF APPEARANCE AND DISAPPEARANCE OF COLD AGGLUTININS IN PRIMARY ATYPICAL PNEUMONIA

A. *First appearance* (36 cases): open bars = last negative test; solid bars = first positive test.

B. *First significant (4-fold or greater) drop in titer* (52 cases): open bars = last observation of the maximum titer; solid bars = first test after the drop in titer.

C. *Disappearance* (39 cases): open bars = last test before a 4-fold or greater drop in titer to 20 or less; solid bars = first test in which the titer was 20 or less (the last of these includes 1 case each on days 71, 87, and 93, respectively).

Cold agglutinins in 2 cases with recurrent attacks of atypical pneumonia

The frequency with which recurrent attacks of primary atypical pneumonia occur is difficult to determine from the available data. In several instances among the present cases, patients were admitted for more than one attack of what was diagnosed as primary atypical pneumonia, but adequate studies during each of the attacks were not made in all of these cases. Two such patients, however, were studied during both attacks and some of the relevant findings are shown in Figures 6 and 7. A resume of some of the clinical features of these cases follows:

Case 88 (Figure 6). This patient was first admitted on January 5, 1943. She had been in fairly good health until 2 weeks previously when she began to have some

² The results of the agglutination tests with this strain are considered in more detail elsewhere (6).

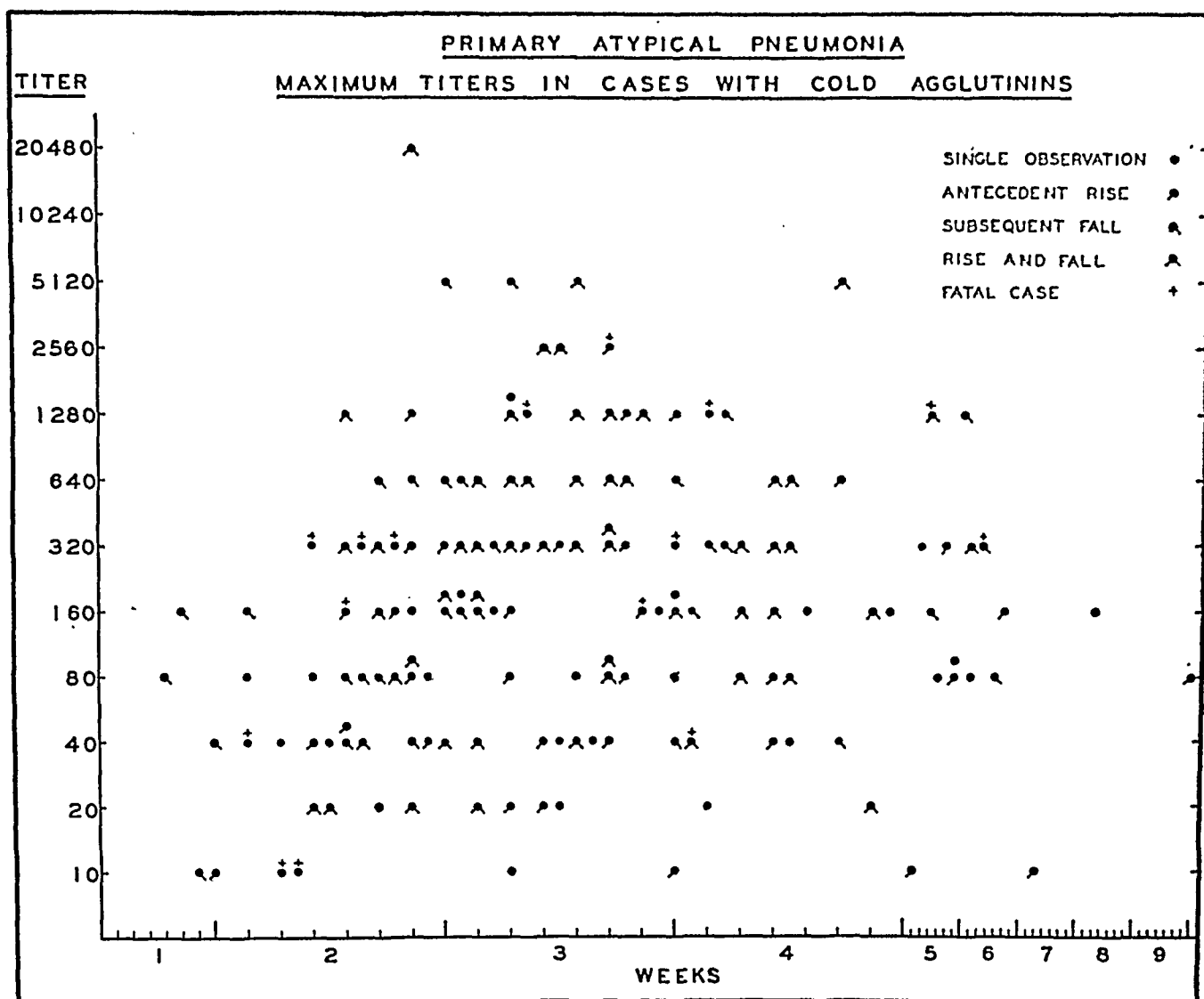


FIG. 2. PRIMARY ATYPICAL PNEUMONIA. MAXIMUM TITERS IN CASES WITH COLD AGGLUTININS

Each dot represents the maximum titer of cold isohemagglutinins in one patient and the time after the onset of the disease when that titer was first demonstrated. A short line sloping up to a dot from the left indicates that observations of lower titers were made in that case before the maximum titer was obtained. A similar line sloping down to the right from the dot indicates that there were subsequent observations of lower titers. Dots without such lines indicate single observations or multiple observations of the same titer within a brief period.

malaise and vomiting. Later on the same day, and again on the following day, she had severe shaking chills. She then began to have a cough which was dry at first and later increased in severity and became productive of thick clear mucoid sputum. She rapidly developed dyspnea which increased in intensity and was associated with moderate cyanosis. Both the dyspnea and cyanosis were markedly aggravated by paroxysms of cough. At the time of admission, she appeared to be extremely dyspneic and cyanotic and showers of râles were made out in the left upper chest both anteriorly and posteriorly. There were also signs of a well developed mitral stenosis although the patient had been unaware of any heart disease. The signs in the lungs spread rapidly so that, within 2 days, râles were heard throughout the entire chest. The

first x-ray on January 9th showed diffuse, finely nodular areas of density throughout both lung fields and closely resembled the radiographic picture of miliary tuberculosis. The patient was given oxygen and during the first 4 days in the hospital, she also received sulfadiazine therapy. Her course was stormy for the first week and was characterized by episodes of extreme dyspnea, cyanosis, and delirium. There was a low grade fever throughout the second week, after the temperature had been normal for 2 days. During this second week, the lungs began to clear and the patient's condition improved steadily. The lungs were essentially clear and the patient was essentially free of symptoms at the time she was discharged on January 29. Cold agglutinins appeared in low titer at the end of the first week of illness, reached a peak of 320

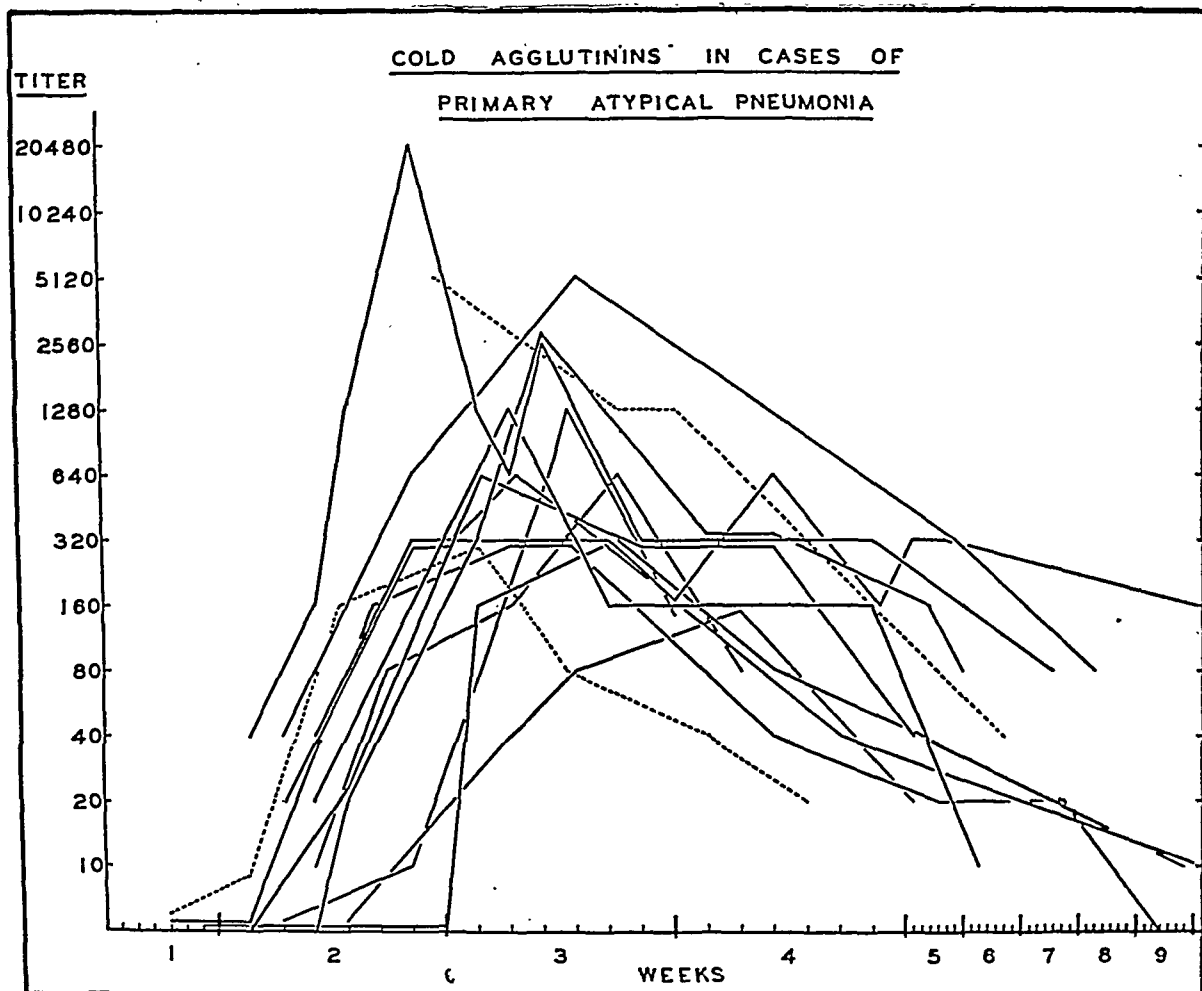


FIG. 3. TITERS OF COLD ISOHEMAGGLUTININS IN A FEW REPRESENTATIVE CASES

The dotted lines represent the observations made during 2 separate attacks in the same patient (Case 88, see Figure 5); the lower one on the left represents the titers observed during the initial attack and the upper one to the right shows the titers obtained during the recurrence.

after another week, and then declined to a level of 20 at the time of discharge. Agglutinins for streptococcus 344 were first demonstrated at the beginning of the third week in the hospital.

The patient was readmitted on November 28, 1943. During the intervening period, she had several episodes of severe dyspnea and cough associated with frequent colds. On November 18, after being free of symptoms for several weeks, she developed severe malaise, a slight sore throat, generalized aches and pains, and severe headache. She then again began to have a cough which was productive of small amounts of thick mucoid sputum. Her coughing spells again made her markedly dyspneic and cyanotic. Two days before entry she noticed a few streaks of blood in her sputum. At the time of admission, the patient appeared to be in extremis. She was markedly orthopneic and cyanotic. Loud, medium, and coarse

crepitant râles resembling those of pulmonary edema were heard throughout both lungs. An x-ray again showed a miliary type of lesion involving most of both lung fields but this time there was slight confluence of the process in the upper part of the right lung in the first film but not in the later ones. She was again given oxygen and sulfonamide therapy for 5 days after which the temperature dropped to normal, but a low grade fever soon recurred. During the middle of the second week in the hospital, the patient developed thrombophlebitis in both lower extremities and was given a second short course of sulfonamide therapy. She again became afebrile for 2 days, after which a low grade fever again recurred. Towards the end of the fourth week in the hospital, the patient had a chill followed by a sharp rise in temperature and she began to have sharp pain in the right lower chest. A blood culture taken at the time of the chill yielded Type 9 pneumo-

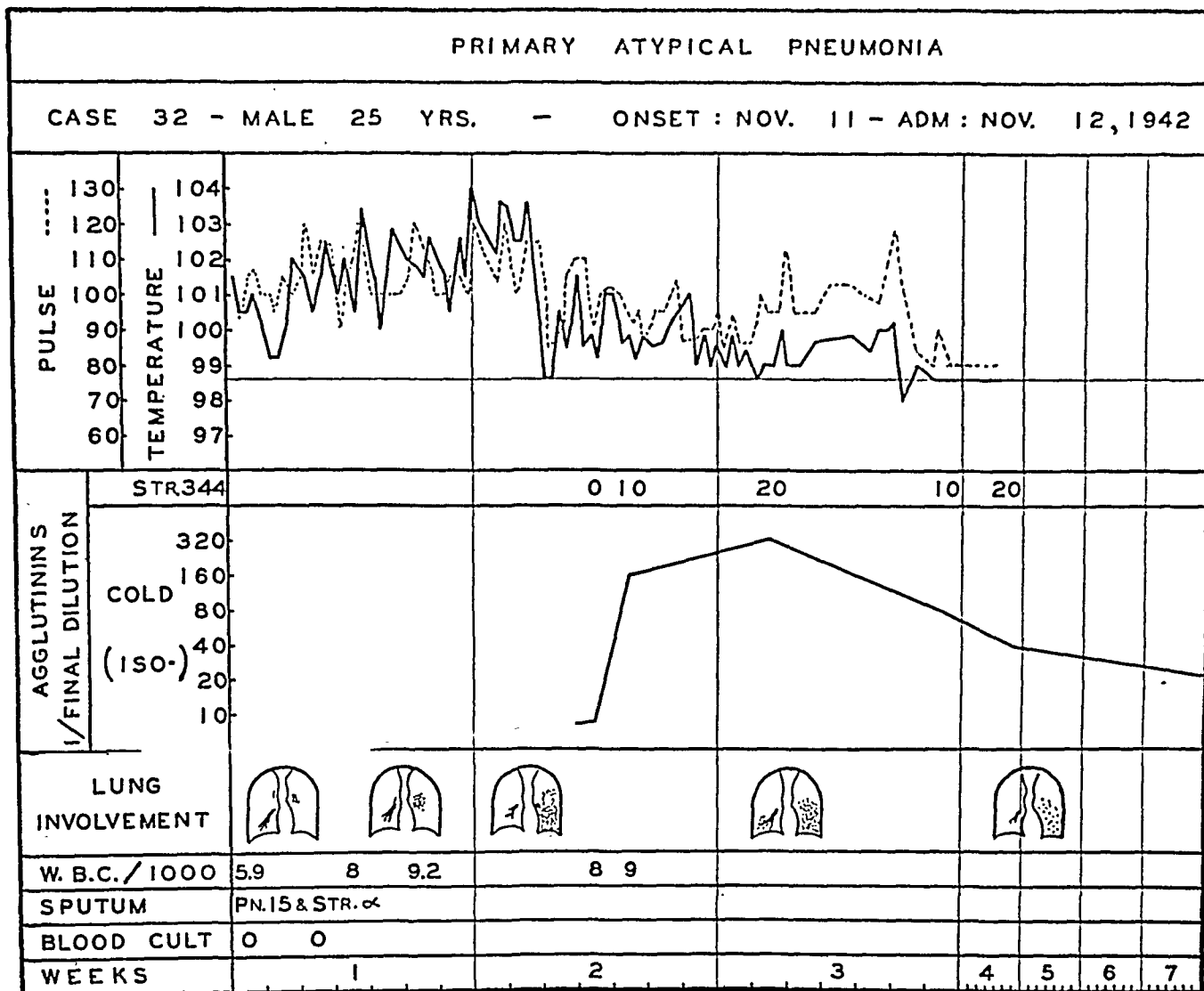


FIG. 4. RELEVANT FINDINGS IN A CHARACTERISTIC CASE OF PRIMARY ATYPICAL PNEUMONIA OF MODERATE SEVERITY

Note. In Figures 3 to 6 inclusive, the horizontal scale begins at the time of admission to the hospital. The following abbreviations have been used: Str. 344 = Streptococcus 344 (the strain of anhemolytic streptococcus described by Thomas et al. (5)). Str. α = streptococcus with alpha hemolysis. Pn. = pneumococcus (the number represents the type). Blood Cult O = no growth in ordinary aerobic blood cultures. W.B.C./1000 = white blood cell count in thousands.

mococcus. Sputum was not obtained until after 2 days of sulfonamide therapy and a culture of some purulent sputum obtained at that time showed hemolytic *Staphylococcus aureus* as the predominant organism. The patient improved after the third day of this episode but full doses of sulfonamides were continued this time for 11 days. Signs of consolidation of the right lower lobe were found by physical examination and x-ray during this episode. The patient improved and the lungs cleared completely before she was discharged on January 4, 1944. Blood was first obtained for cold agglutinins at the end of the second week of this attack and a titer of 5120 was found at that time. Thereafter, the titer dropped gradually to 80 in the course of the next 4 weeks. Agglutinins for streptococcus 344 were found in the first blood in a titer of 80 but none were demonstrated in later specimens.

Case 102 (Figure 7). The first admission in this case was on April 11, 1943. Ten days previously, this patient began to have anorexia, alternating fever, chilly sensations, and prostration. On the next day, he had severe frontal headache and slight sore throat. His physician prescribed sulfadiazine which he took for 4 days without affecting his symptoms or fever. At the time of admission, he was only moderately ill without dyspnea or cyanosis. There were some patchy ulcerations on his tonsils and fine diffuse injections of his throat. He coughed frequently and raised scanty amounts of thick gray mucoid sputum. Showers of medium crepitant râles were heard over the middle of the left chest anteriorly and in the axillary region on the same side. An x-ray taken at this time showed a finely mottled infiltration of the middle half of the left lung field. The patient continued to have

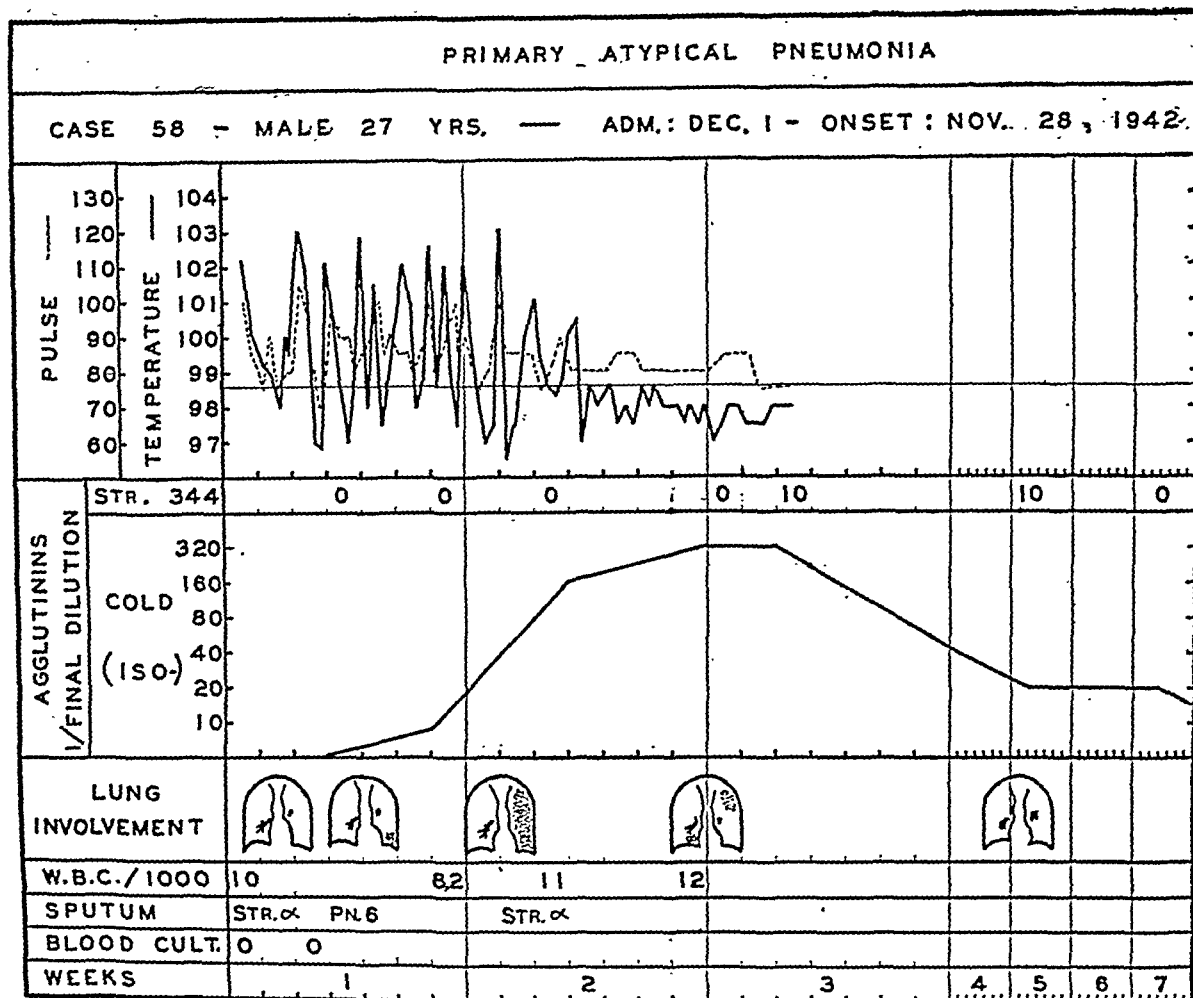


FIG. 5. RELEVANT FEATURES OF THE COURSE IN A CHARACTERISTIC CASE OF PRIMARY ATYPICAL PNEUMONIA OF AVERAGE SEVERITY

The patient had been intimately exposed to the one whose course is shown in Figure 4 (see note under Figure 4).

fever and severe cough during the first 4 days in the hospital. The fever then subsided and the cough improved gradually although the pulmonary process extended to involve a large part of the right lower lobe. The clinical condition of the patient, however, continued to improve and the lungs were entirely clear at the time of discharge. Cold agglutinins in a titer of 40 were found in the first blood taken on the day after admission which was the eleventh day of the disease. The maximum titer during this admission was 640 and was obtained one week later after which the titer dropped steadily to a level of 40 at the time of discharge. Agglutinins for streptococcus 344 were not demonstrated in the first blood but were found in a titer of 40 during the next 2 weeks. This patient was a pigeon fancier and he had raised pigeons for some time prior to this illness. He was, therefore, suspected of having ornithosis. All of the samples of his serum were sent to Dr. Karl F. Meyer for complement fixation tests with psittacosis virus. The tests were entirely negative.

The second admission of this patient was on October 17, 1943. He had been perfectly well from the time of discharge until October 3 when he suddenly began to have a severe hacking cough productive of thick yellow mucoid sputum. Shortly thereafter, he developed substernal soreness and a sense of oppression in the chest, particularly associated with coughing. He also had a slight sore throat and headache. Two days before entry, he began to have chilly sensations, generalized aches, malaise, slight nausea, and an increase in his fever. The physical examination and x-ray of his lungs at this time were essentially the same as those found at the time of earlier admission, except that a few rales were heard in the right lung at this time. His lesion extended to involve most of the right lower lobe. He received sulfanamide therapy during the first 6 days in the hospital. His fever subsided on the third day and his lungs began to clear by the end of the first week in the hospital. At no time did he appear very ill nor did he have any dyspnea or cyanosis.

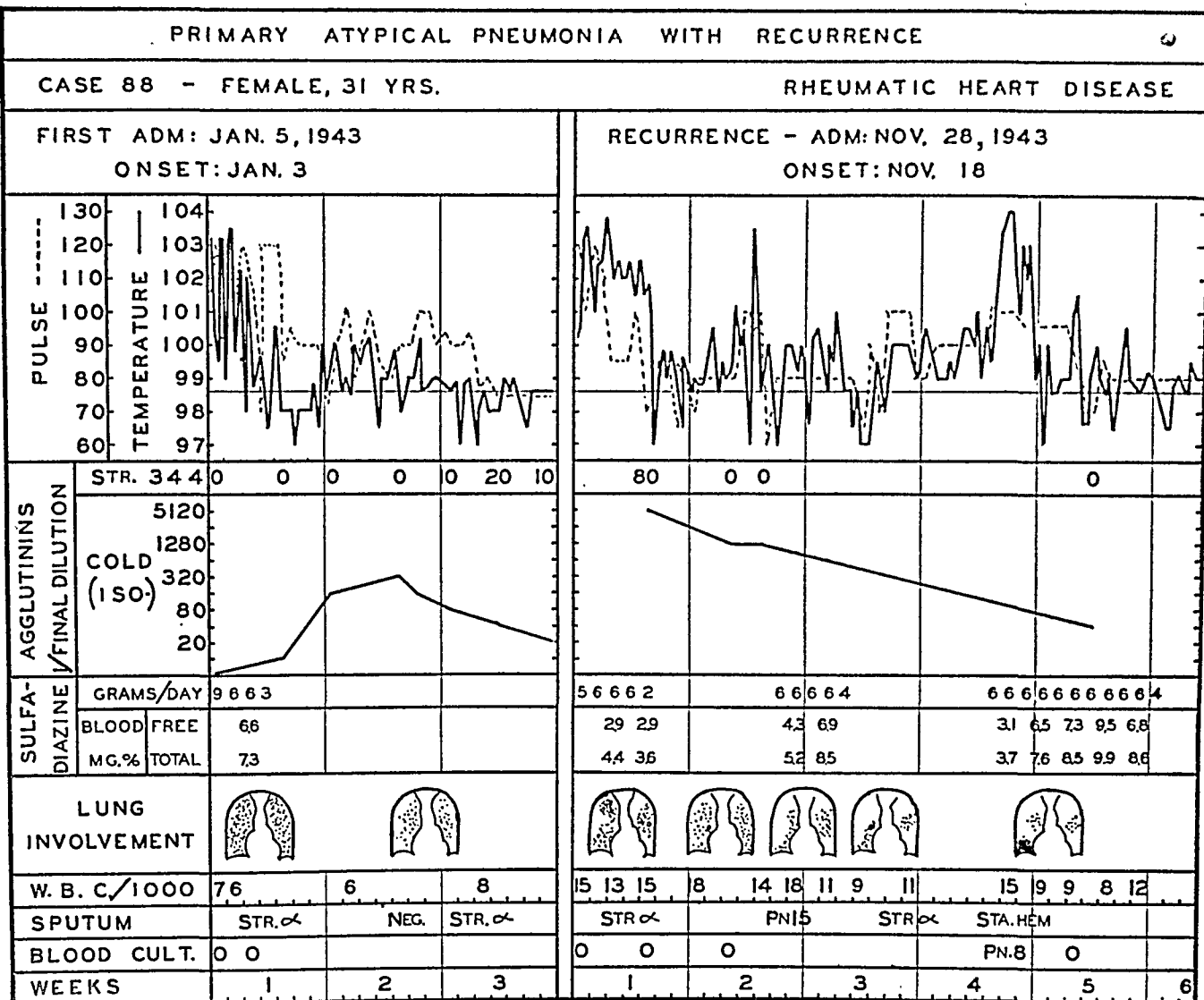


FIG. 6. RELEVANT FINDINGS IN THE COURSE OF 2 ATTACKS OF PRIMARY ATYPICAL PNEUMONIA IN A PATIENT WITH RHEUMATIC HEART DISEASE AND MITRAL STENOSIS

There were no complications during the first attack. In the recurrent attack, the patient had bilateral thrombophlebitis of the legs during the second and third hospital weeks and Type 8 pneumococcal pneumonia, probably following a pulmonary infarct, during the latter half of the fourth week in the hospital (see note under Figure 4).

At the time of discharge, his lungs were entirely clear. Cold agglutinins were not found in his blood at the time of admission which was considered to be the fourteenth day of his illness. They appeared in comparatively low titer during the next 10 days and again dropped off in the following week. Agglutinins for streptococcus 344 were present in all specimens tested.

Factors influencing the occurrence and titers of cold agglutinins

These last cases and the 2 previous ones serve to illustrate what has already been said about the appearance and course of the cold agglutinins in most of the cases of atypical pneumonia. The

impression was gained in the course of observing the patients that the occurrence of cold agglutinins and the height to which the titers rose was related to the severity of the disease. On the other hand, observations in a number of individual cases did not seem to bear this out. It was of interest, therefore, to analyze the data further with reference to the relation of the occurrence and maximum titers of cold agglutinins to some of the important factors in the disease. First, an attempt was made to correlate the maximum titers with the general impression of the severity of each case as it was gathered from a study of the course and

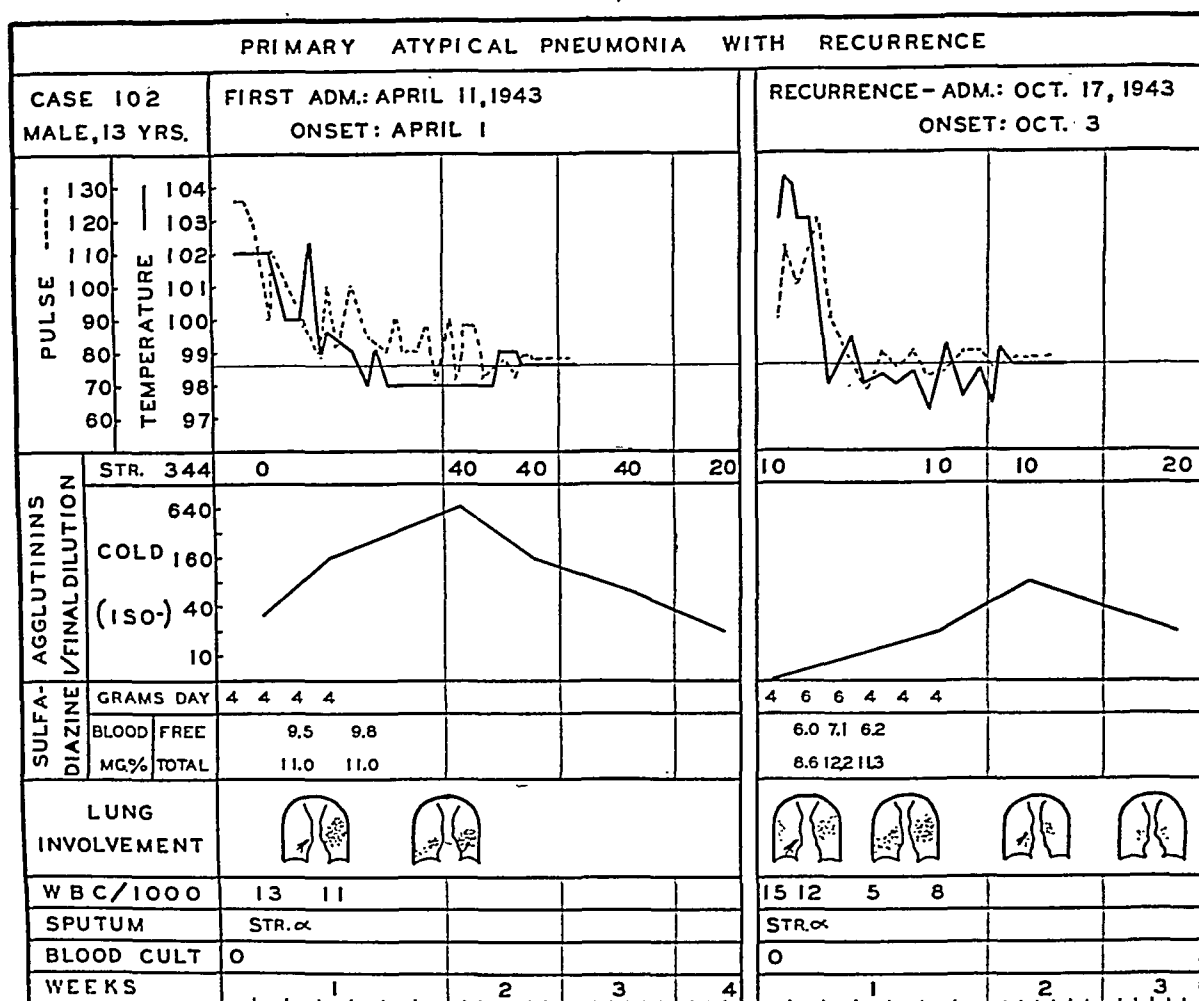


FIG. 7. RELEVANT FEATURES OF THE COURSE IN 2 ATTACKS OF ATYPICAL PNEUMONIA IN THE SAME PATIENT
There were no complications in this case (see note under Figure 4).

symptoms and all other available data. More detailed analyses were also made of specific features of the disease such as the extent of the pulmonary lesion, the height and duration of the fever, the leukocyte count, the age of the patient, and the administration of sulfonamide drugs.

Severity of the disease. Based on the clinical findings, an arbitrary grading of severity from I to V was given to each case. While it may be difficult to compare these grades accurately with the severity of the cases reported by others, this grading will serve to compare the relative severity in the different groups of cases in the present study. Roughly, Grade I corresponds to the severity of the large majority of the cases which have been reported by observers among ado-

lescents and young adults in schools, colleges, and most military hospitals. Grades II and III correspond to the severe or the moderately severe among those cases. Grades IV and V represent the severest types of cases. The relation of the severity of the disease thus graded to the occurrence and the maximum titers of cold agglutinins is shown in Table I.

The 45 cases in which cold agglutinins could not be demonstrated and the 18 in which they were found in a titer of only 10 or 20 include most of the cases in which studies were inadequate. In about two-thirds of them, there were no observations between the 12th and 30th day of the disease. Since cold agglutinins may make their first appearance after the 12th day and may

TABLE I

Primary atypical pneumonia. Relation of cold isohemagglutinin titer to the severity of the disease

Grade of severity	Maximum cold agglutinin titer						Totals
	Less than 10	10-20	40-80	160-320	640-1280	2560 or higher	
I	26(20)	5(2)	4	4	0	0	39
II	3(3)	5(3)	15	9	2	0	34
III	9(8)	6	20	18	7	1	61
IV	6(5)	1	3	15	14	2	41
V-R	0	0	2	2	2	4	10
V-D	1(1)	1(1)	2	7	3	1	15
Totals	45(37)	18(6)	46	55	28	8	200

R = Recovered. D = Died.

Parentheses enclose the number of cases with titers less than 20 in which no observations were made between the 12th and 30th day of the disease.

reach low levels by the end of the fourth week after higher levels had been reached earlier, it cannot be said for certain that these patients did not have cold agglutinins during that interval. Omitting these cases, there were only 6 of Grade I severity without cold agglutinins and 3 with a maximum titer of 10 or 20. Similarly, among the cases of Grades II, III, and IV combined, there were only 2 without cold agglutinins and 9 in which the highest titer was only 10 or 20. Among the fatal cases, the last observation was made on the fifth day in the case without cold agglutinins and on the ninth day in the one with the cold agglutinin titer of 10.

Allowing for these facts and for the probability that most of the deaths occurred before the maximum titers could be attained, there is a definite correlation between the severity of the disease and the maximum observed cold agglutinin titer. The greater the severity of the disease, the greater is the proportion of cases in which the cold agglutinin titer reached high levels. Omitting fatal cases, titers of 640 or higher were not observed in any of the cases of Grade I severity; and were demonstrated in 6, 13, 39, and 60 per cent of those of Grades II, III, IV, and V, respectively.

Extent of the pulmonary lesion. In most of the cases, the severity of the symptoms were largely dependent on the amount of lung involved, but there were many discrepancies. The relation of the maximum titer of cold agglutinins to the amount of lung involved, as estimated from the

physical and x-ray findings, is shown in Table II. There was a close though not complete correlation between the number of lobes involved and the maximum observed cold agglutinin titers.

The case reported by another worker (7) is of interest in this connection. In that case, the symptoms were those of a very severe atypical pneumonia without abnormal physical signs and with x-ray findings consistent with tracheo-bronchitis, but no areas of consolidation or infiltration were noted. High titers of cold agglutinins were noted on the 15th to the 23rd days. Other authors (15) also found a titer of 1000 in a girl who had a severe cough but was afebrile and ambulatory and had a negative x-ray.

Age. From the fact that most of the early reports concerning the disease now generally recognized as primary atypical pneumonia were from

TABLE II

Primary atypical pneumonia. Relation of the cold isohemagglutinin titer to the extent of the pulmonary lesion

Number of lobes involved	Maximum cold agglutinin titer						Totals
	Less than 10	10-20	40-80	160-320	640-1280	2560 or higher	
1 or less	19	7	12	15	4		57
2	20	7	15	18	5	2	67
3	4	2	13 ¹	7 ¹	7 ¹	1	34 ³
4		1	1	2	3		7
5	2 ¹	1 ¹	5 ¹	13 ³	9 ²	5 ¹	35 ¹²
Totals	45 ¹	18 ¹	46 ²	55 ⁷	28 ³	8 ¹	200 ¹⁵

Superscripts denote number of fatal cases included.

schools and military hospitals, the impression generally prevails that the disease is limited in its occurrence to adolescents and young adults. In the present series, to be sure, there was a predominance of cases in young adults but there was also an appreciable number in persons over 40 and, indeed, over 60 years of age. Since, in other kinds of pneumonia, the severity of the disease, at least as judged from case fatality rates, is usually closely related to the age of the patients, it was of interest to see whether the cold agglutinin titers in the present series of cases were related to this factor. The data are shown in Table III and indicate no definite correlation between the age of the patients and the maximum cold agglutinin titers.

It is interesting to note in passing that there was a steady increase in mortality in the increasing age groups. The rise in mortality for each decade, however, was far less than that which is usually observed in cases of bacterial pneumonia.

Height and duration of fever. The relation of these findings to the maximum cold agglutinin titers is shown in Table IV. The highest temperatures for purposes of this table, were recorded on more than one day, or at least for several readings throughout a single day, during the primary disease, and without relation to complicating bacterial infections. If those cases are excluded in which cold agglutinins were not demonstrated—and this seems reasonable from what has already been said—there appears to be a definite relation between the maximum temperatures and the cold agglutinin titers. The higher the temperatures, the greater was the proportion of cases with high cold agglutinin titers. Likewise, the higher the maximum cold agglutinin titers, the greater was the proportion of cases in which the higher temperatures were attained. There is a closer correlation, however, between the duration of the fever and the maximum cold agglutinin titers observed. The longer the fever persisted, the greater was the proportion of cases having high titers, but this was less striking in cases with fever of more than 3 weeks' duration.

Leukocyte counts. The relation of the leukocyte counts to the maximum cold agglutinin titers observed is also shown in Table IV. In about one-half of the cases, the leukocyte counts were essentially normal or only slightly elevated (7500 and 12,500). In most of the remaining cases, they remained below 7500 throughout most of the febrile course and in only 19 cases were counts above 12,500 observed during the acute disease. In many cases, particularly in severe ones, the count rose from low or normal levels to higher ones, usually during the latter part of the illness.

Omitting the cases without cold agglutinins, there is a definite though not very close relation between the level of the leukocyte counts and the maximum observed cold agglutinin titers. The higher the leukocyte count, the greater was the proportion of cases with high cold agglutinin titers. Rises in the counts were noted more often among the cases with high cold agglutinin titers.

TABLE III

Primary atypical pneumonia. Relation of the cold isohemagglutinin titer to the age of the patient

Age group (years)	Maximum cold agglutinin titer						Totals
	Less than 10	10-20	40-80	160-320	640-1280	2560 or higher	
19 or less	8	6	18	13	11 ¹	1	57 ¹
20 to 29	8	5	15 ²	17 ¹	5		50 ³
30 to 39	13 ¹	3	5	11 ²	9	4	45 ³
40 to 49	10	2 ¹	7	4 ²	2 ¹	2	27 ⁴
50 to 59	4	1		5	1 ¹	1 ¹	12 ²
60 or more	2	1	1	5 ²			9 ²
Totals	45 ¹	18 ¹	46 ²	55 ⁷	28 ³	8 ¹	200 ¹⁵

Superscripts denote number of fatal cases included.

Sulfonamide therapy. There are several reports of acute hemolytic reactions associated with sulfonamide therapy (8 to 11). Sulfadiazine and sulfathiazole were the drugs implicated in recent cases, but such reactions were more frequent in sulfanilamide-treated cases. The case histories in many instances were quite characteristic of atypical pneumonia but data are not available concerning cold agglutinins in the blood before the development of the anemia. There are, however, reports of anemia and cold autohemagglutinins occurring in pneumonia without relation to sulfonamide therapy (12, 13).

The cases of the present series are listed in Table IV according to the amounts of sulfonamide therapy administered during the acute disease. One-fourth of all the cases received no sulfonamide at all throughout the course of their illness. In the remaining cases, varying amounts were given either because of the failure of the patients' fever and symptoms to improve or because bacterial complications were demonstrated or suspected. There was no striking difference between the distribution of the maximum titers of cold agglutinins in those cases in which various amounts of sulfonamides were used as compared with those in which such drugs were withheld. Furthermore, many of the high titers were obtained before sulfonamide therapy was started.

Time when the cases occurred. It was of interest to see whether cold agglutinins were encountered more often or in higher titers at one time of the year as compared with another. The numbers of cases observed each month are shown in Figure 8 and the maximum titers are as pro-

TABLE IV

Primary atypical pneumonia. Relation of maximum cold isohemagglutinin titer to the height and duration of fever, to the leukocyte count, and to sulfonamide therapy

Maximum titer	Less than 10	10-20	40-80	160-320	640-1280	2560 or higher	Totals
Maximum temperature							
Less than 101° F.	15	5	7	13	3	0	43
101 to 102.9	6	2	14	18	5	1	46
103 or higher	24	11	25	24	20	7	111
Totals	45	18	46	55	28	8	200
Duration of fever							
7 days or less	18	2	5	1	0	0	26
8 to 14 days	15	11	25	22	4	2	79
15 to 21 days	6	5	10	25	19	4	69
22 or more days	6	0	6	7	5	2	26
Totals	45	18	46	55	28	8	200
Leukocyte count							
Less than 7500	22	8	20	17	7	2	76
7500 to 12,500	18(8)	10(1)	22(9)	33(11)	18(2)	4	105(31)
12,600 or more	5(6)	0(4)	4(9)	5(8)	3(8)	2(4)	19(39)
Totals	45	18	46	55	28	8	200
Sulfonamide therapy							
None	18	3	11	15	3		50
5 to 20 grams	5	4	10	14	4	2	39
21 to 40 grams	14	6	13	14	14	3	64
41 or more grams	8	5	12	12	7	3	47
Totals	45	18	46	55	28	8	200

Parentheses enclose the numbers of additional cases in whom leukocyte counts reached these levels late in the course of the illness after previous lower counts.

piately indicated in each instance. There was no definite correlation between the maximum titers and the time when the cases occurred.

The monthly incidence of cases may be noted in passing. There were 2 waves: the first and larger of them in the fall and winter of 1942-43 and the smaller during the same season of the following year. Deaths were most frequent in October 1942. Characteristic cases of primary atypical pneumonia were rarely encountered during the epidemic of influenza which reached its peak in December 1943 or in the months which followed. Similar curves of incidence have been noted by other workers during the same period.

Some of the more important relationships which have been discussed are summarized in Table V. Briefly, the maximum cold agglutinin titer is related quite closely to the general severity of the symptoms, to the number of lobes involved, and to the duration of fever. Less striking is its relation to the height of the fever and to the leukocyte count. There is no correlation between the

cold agglutinin titer and the age of the patient, the administration of sulfonamides, or the time when the cases occurred.

Hemolytic anemia in atypical pneumonia and its relation to cold agglutinins and to sulfonamide therapy

The original observations concerning the occurrence of cold agglutinins in atypical pneumonia were made in September 1942 following the occurrence, in rapid succession, of 2 cases of acute hemolytic anemia. Both of them were extremely ill and their entire lungs were involved. In one of them, sulfathiazole and sulfadiazine had been given for several days before the anemia was recognized; in the other, there was marked anemia before the first and only dose of sulfadiazine was given. In each instance, the presence of cold agglutinins was first indicated by difficulties in blood grouping and cross matching preliminary to transfusion and also was indicated by the appearance of clumping during red blood cell

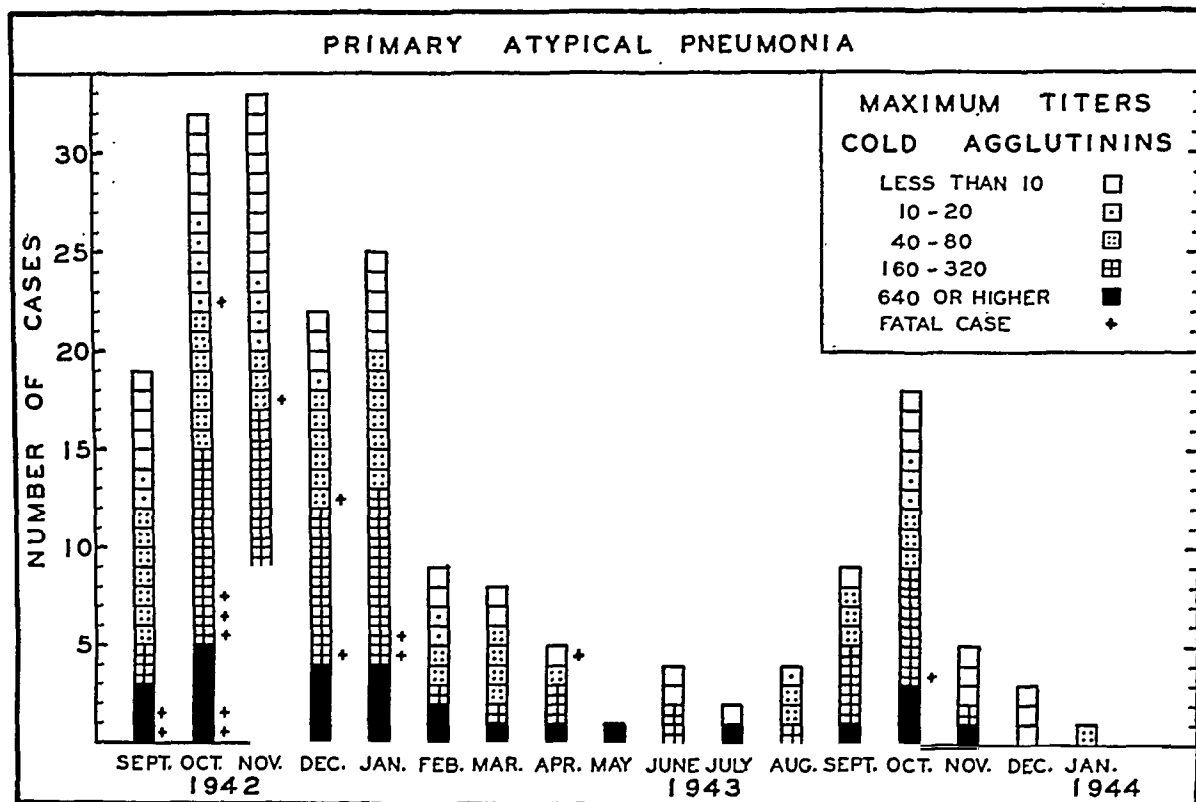


FIG. 8. THE MONTHLY OCCURRENCE OF THE 200 CHARACTERISTIC CASES OF PRIMARY ATYPICAL PNEUMONIA INCLUDED IN THIS STUDY

Each square represents a single case and the cold isoheamagglutinin titer is appropriately designated.

counts. In the first of these cases, the titer of cold agglutinins tested with the cells of another group O donor was 640. This test was done at least 3 days after the anemia had developed, and sulfadiazine was still being given. In the second patient, there was no opportunity to do such quantitative studies and this case is not included in the present series. Both of these patients died, the first one several days after the anemia was recognized and the other after only a few hours. Autopsies were done in both cases and revealed characteristic pulmonary lesions which will be described elsewhere.

In the present series, there were a total of 11 cases in which various degrees of anemia were demonstrated. Most of the severe ones were first recognized only after the anemia was well established so that accurate observations concerning the development of this condition were not available. A few relevant observations which are available in these cases will be noted. More de-

tailed studies concerning the mechanism of anemia were made in some of the cases by Dr. T. H. Ham and Miss Geneva A. Daland but these are not included here.

In 2 cases, a drop in hemoglobin of 15 to 20 per cent was demonstrated. In one of them, the drop occurred early in the third week of the disease during the first days of sulfadiazine therapy and the drug was continued without aggravating the anemia appreciably. In the second, the anemia developed 1 week after sulfadiazine therapy was stopped. In the former, the cold agglutinin titer was 4096 and in the latter 5120, both being noted at the height of the disease. These titers dropped in both instances to a level of 80 over the following 6 weeks.

In 5 cases, a drop in hemoglobin of between 20 and 40 per cent was noted. In 3 of them, the anemia was fully developed by the end of the second week of the disease and sulfonamide therapy was started later. The blood hemoglobin increased during this treatment. In the other 2 cases, the anemia occurred during the course of sulfonamide therapy: in one case, in the second week, and in the other, during the fourth week of the disease. In these patients, also, the sulfonamide therapy was continued after the anemia occurred, and the hemoglobin levels rose to

TABLE V

Primary atypical pneumonia. Summary of some factors influencing the cold isohemagglutinin titer

Maximum cold agglutinin titer	Less than 10	10-20	40-80	160-320	640-1280	2560 or higher	Totals
Number of cases *	45	18	46	55	28	8	200
Severity, III to V	36	44	59	76	93	100	61
4 or 5 lobes involved	4	11	13	27	46	63	21
Temperature 101° F. or higher	67	72	85	76	89	100	74
Fever 15 days or longer	27	28	35	58	86	75	48
Leukocytes, <7500	49	44	43	31	25	25	38
Sulfonamide therapy	60	83	76	73	89	100	75
Age, 30 years or older	64	39	28	45	43	88	52

* Figures below this line are percentages of these numbers.

spite of that fact. Sulfadiazine was the drug used in 4 of these cases and sulfathiazole was used in the fifth. In 2 of the cases, the titer of cold isohemagglutinins was only 40 shortly after the anemia was recognized. In the third case, the titer was 320, and in the fourth 640.

In one of the patients, cold agglutinins could not be demonstrated in many specimens of serum by the usual method, that is, with the red blood cells from another group O donor. There was, however, definite and quite marked autoagglutination demonstrated in the cold and this was reversible on warming. This was noted in the clumps of erythrocytes seen during the blood counts and also in the whole oxalated blood which showed the red blood cells agglutinated as a hard single mass when removed from a refrigerator and a normal suspension of the red cells when the same blood was warmed in an incubator. The titer of autoagglutinins, however, was not quantitated. Agglutinations were done with erythrocytes from several group O donors and were all negative. This case may, therefore, represent an instance in which there were cold autohemagglutinins without cold isohemagglutinins. It is possible also, that, in some of the other cases, the autohemagglutinin titers may have been considerably higher than the titers demonstrated with another donor's cells. In a few other sera in this study and in 7 of Turner's (14), the results of the 2 tests were in essential agreement.

In the 4 remaining patients, the anemia was severe and the hemoglobin dropped 40 to 70 per cent in each case. This usually occurred over a period of 3 days or less. In 1 of the cases, the anemia was first recognized during the second week of the pneumonia and the cold isohemagglutinin titer at that time was 1280. Sulfadiazine was given later and the anemia improved during this therapy. In the other 3 patients, including the earliest case that was recognized, the anemia was first noted towards the end of the third week of a very severe illness in which almost the entire lung was affected. Sulfonamides had been given for several days in each instance. Sulfadiazine was

used in one, sulfathiazole in another, and both drugs in the third. The first test for cold agglutinins was done in each instance after the anemia was established and the titers at that time were 640, 1280, and 5120, respectively.

Some evidence for the hemolytic nature of the anemia was obtained in most of these cases. The drop in hemoglobin was usually accompanied by leukocytosis, with total white blood cell counts rising to 20,000 or higher. In some, the icterus index was later elevated and reticulocytosis was noted at that time.

Although these data are inadequate in many respects, they nevertheless suggest that the hemolytic anemias observed in these cases of primary atypical pneumonia were in some way related to the presence of cold agglutinins. While it cannot be denied that either sulfadiazine or sulfathiazole, the drugs used in these cases, may under certain circumstances themselves be responsible for the occurrence of acute hemolytic anemia, the evidence in the present cases suggests that these drugs were probably not the cause of the anemia nor did their administration appear to aggravate the condition when it occurred.

DISCUSSION

It was the main purpose of this paper to characterize the relationship of cold agglutinins to atypical pneumonia. The time in the disease when they make their appearance and the course of the cold agglutinins are suggestive of an antibody response as has been postulated (14, 15). In that event, it must be assumed that the antigen

involved is in some way related to the etiological agent of the disease or to the products of its action on the human body during the course of the infection. This hypothesis is consistent with the correlation of the high titers with the severity of the disease, the extent of the pulmonary lesions, duration of the fever, and also with the more frequent findings of cold agglutinins in patients who had elevated leukocyte counts.

A number of the properties of the hemagglutinins in some of these cases were studied. They were found to be similar to others described (12, 14 to 16). Certain of these findings and a consideration of some of the features of the test for cold agglutinins will be presented in subsequent papers.

Agglutinins for streptococcus 344 (5) were noted in connection with the findings in some of the representative cases presented in the text and in Figures 3 to 6. Almost all of the patients with atypical pneumonia who had cold agglutinins in significant titers were also found to have agglutinins for this strain of streptococcus. The details of these findings will be considered elsewhere. The findings are of interest because they suggest that there is also an antigen in this streptococcus which is in some way related to the etiologic agent in the present cases.

An alternative hypothesis concerning the nature of the cold agglutinins in primary atypical pneumonia might be considered. This hypothesis would attribute to the unknown virus of this disease, 2 characteristics which are common to some, and possibly to many other viruses. The first is the property of certain virus particles or their soluble products to agglutinate human erythrocytes and those of other animal species, as exemplified by the agglutination of the erythrocytes of fowl, of humans, and of other animal species by the various known types of influenza virus—the now well known Hirst phenomenon. The other is the common property of certain viruses to persist in the body of the host for long periods after all evidence of active infection has disappeared. On the basis of these 2 properties of viruses, one might assume that the cold agglutinin is the result of the interaction of red blood cells with the virus or its products.

This hypothesis would seem to be inconsistent with the fact that cold agglutinins do not appear

until several days after the onset of symptoms. On the other hand, if it is correct, one could assume that virus activity takes place and causes infection in some part of the body for several days before the virus particles or their products are discharged into the circulation. Such an hypothesis would be consistent with the increase in the titer of cold agglutinins towards the end of the disease. It would also be consistent with the rapid drop in titer and the early disappearance of the cold agglutinins in most of the cases. In addition, it would account for the high titers observed in the severe cases and the persistence of the cold agglutinins in many such cases. Clinically, it is known that some of the severe cases of primary atypical pneumonia are followed by symptoms which persist for a long time and suggest continued infection. Notable are the asthmatic-like attacks which in occasional cases occur for several weeks after the acute disease has subsided. These attacks and the lassitude and general weakness which are common after severe attacks, may be the results of persistent low grade infection.

The mechanism of the anemia in the present cases is of interest. It occurred in each case after the middle of the second week of the acute disease at a time when maximum titers of cold agglutinins are most often encountered. The anemia was apparently independent of sulfonamide therapy and not affected by it. The mechanism of its occurrence is a matter for speculation. From the results of *in vitro* studies by others (17 to 19), the agglutination of erythrocytes renders them more susceptible to hemolysis when subjected to mechanical stimuli. On the basis of their observations, certain of these authors (19) also suggest that cohesion of erythrocytes may lead to their increased mechanical destruction while in motion in the circulation. Their observations have shown this condition to occur in the presence of isoagglutinins and cold agglutinins and with sickled cells. Further unpublished studies by Ham, in a patient with cold agglutinins who had a negative Donath-Landsteiner test, indicated that hemoglobinemia could be produced by exposure of a limb to the cold. In patients with atypical pneumonia, particularly during the height of the disease and in severe cases, there may be transient reduction in the skin temperature, especially of the extremities. This may occur during chilli-

or as a result of exposure or of the administration of antipyretics. In patients with marked cold autoagglutination, there is usually a wide thermal range and significant agglutination may still occur at the temperatures which are attained in the skin of the extremities under such conditions. This might conceivably represent the conditions under which hemolysis might occur through the mechanism suggested above (19).

Tests for autohemagglutination unfortunately were not carried out in many of the cases. In the few that were done in this study and by others (15), there was essential agreement between the autohemagglutinin and the isohemagglutinin titers. There is reason to believe, however, that in certain of the patients with atypical pneumonia, autohemagglutinins may develop and not be demonstrable by the tests for isohemagglutinins—that is, with the use of erythrocytes from other donors. An example of such a case which was complicated by a hemolytic anemia was presented. Similar observations were made in one other case without hemolytic anemia in which agglutination of the patient's own cells was marked and occurred in high titers but could not be demonstrated by the method used in the tests which employed cells from other donors. This suggests the possibility that the antigen responsible for the cold agglutinins may be related directly to the tissues of the infected patient.

SUMMARY AND CONCLUSIONS

An analysis has been presented of the results of tests for cold isohemagglutinins in 200 characteristic cases of primary atypical pneumonia of unknown etiology which occurred between September 1942 and January 1944. On the basis of these findings, the relation of the cold isoagglutinin to atypical pneumonia may be characterized as follows:

Cold agglutinins are absent early in the disease. They usually make their appearance during the second or third week after the onset of symptoms and the titers increase rapidly thereafter. The maximum titers are attained in most instances between the middle of the second and the middle of the fourth weeks. The height of the maximum titer appears to be unrelated to the time when it is attained. The titers drop fairly rapidly after reaching the maximum so that significantly lower

ones are already found between the third and fifth weeks. In most of the cases, the cold agglutinins can no longer be demonstrated in a significant titer by the fourth to the sixth weeks. Significant titers persist longer in cases in which the maximum titers are very high.

The maximum titers observed by the method employed ranged from 10 to 10,000. In most instances, the highest observed titers were between 40 and 1280. The height to which the cold agglutinin titers rose was related in a general way to the severity of the symptoms, to the extent of the pulmonary lesion, and to the duration and height of the fever. The maximum titers were also somewhat higher in cases in which there was a leukocytosis during some part of the acute disease, usually during the latter part of the febrile course. The height to which the cold agglutinin titer rose was unrelated to the age of the patient, the administration of sulfonamides, or the time of year when the case occurred.

Some of the relevant findings in 11 cases of hemolytic anemia of varying severity which occurred among these 200 cases of atypical pneumonia were presented. The relation of the anemia to the cold agglutinins and to sulfonamide therapy was discussed. It was suggested that under certain circumstances agglutination may occur in the peripheral circulation. The agglutinated erythrocytes are then subjected to mechanical destruction and thus give rise to hemolysis and anemia.

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COLD AGGLUTININS. III. OBSERVATIONS ON CERTAIN SEROLOGICAL AND PHYSICAL FEATURES OF COLD AGGLUTININS IN CASES OF PRIMARY ATYPICAL PNEUMONIA AND OF HEMOLYTIC ANEMIA

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Some of the properties of cold agglutinins in certain human sera have been listed in a recent review (1). The knowledge concerning these properties is based on studies of the phenomenon by various methods in serum or plasma from isolated cases of a large variety of conditions. With respect to the cold agglutinins in cases of primary atypical pneumonia, limited studies of certain aspects were reported in 1918 (2) in one case of atypical bronchopneumonia, in 1938 (3) in a similar case, and recently (4) in a few cases which more closely resemble those of primary atypical pneumonia of unknown etiology with which we have been concerned in the previous papers of this series.

In the course of serological studies of cases of atypical pneumonia beginning in the fall of 1942, a number of observations were made on certain features of the cold agglutinin reaction and on the methods used for demonstrating and titrating these agglutinins. Circumstances did not permit extensive and complete studies of all the aspects of this reaction which were contemplated. A number of the observations that were made are of sufficient interest, however, to warrant a presentation of the results at this time, even though some of them are only of a preliminary nature. The investigations which are reported in this paper deal with two broad categories: (1) some serological properties of the cold agglutinins, particularly their relation to the human group isoagglutinins and to cold agglutinins for erythrocytes of other animals, and (2) certain physical properties of the cold agglutinins. Data concerning certain features of the technique are presented in the paper which follows.

MATERIALS AND METHODS

Sera. The sera used in the present study were obtained from 3 types of individuals: (1) a number of pa-

tients with primary atypical pneumonia that were included in the previous study (5); (2) patients with hemolytic anemia associated with cold agglutinins but without atypical pneumonia (these cases have also been referred to in the previous studies (6)); and (3) patients and members of the hospital staff who were free of respiratory disease and of blood dyscrasia. Venous blood was drawn and cell-free serum was obtained by centrifugation after the clotted blood had been warmed at 37° C. for at least an hour. Some of the sera had been stored for various periods up to 6 months before the studies were made.

Erythrocytes. Blood was obtained from normal human donors by venepuncture and from animals either by cardiac or venous puncture with either a citrate or oxalate anticoagulant. The blood cells were washed 3 times in a large volume of physiological saline and then resuspended in saline.

The technique of the cold agglutination tests was the same as that employed in the previous studies of this series. Deviations from this method will be mentioned specifically along with other details of methods used in each of the experiments.

COMPARISON OF TITERS IN SERUM AND PLASMA

On a number of occasions, clotted and oxalated samples of blood were obtained at the same time in cases of atypical pneumonia and hemolytic anemia. Titrations of the cold agglutinins were carried out simultaneously on the serum obtained from the clotted blood and on the plasma removed from the corresponding sample of oxalated blood. Identical titers were obtained in every instance. Almost all of the tests to be reported in this paper were carried out on sera removed from clotted blood.

RELATION OF COLD AGGUTININS TO THE BLOOD GROUP SPECIFIC ISOAGGLUTININS

In carrying out the tests for cold agglutinins, group O cells were used routinely in order to avoid agglutination resulting from the group specific agglutinins. In a number of the previous

TABLE I

Effect of absorption of isohemagglutinins (with Group AB erythrocytes) on the cold agglutination of erythrocytes of all 4 major blood groups

Serum	Blood group of patient	Unabsorbed		After absorption with equal volume of washed and packed AB cells at 37° C.									
		Microscopic agglutination		Microscopic agglutination		Macroscopic agglutination							
						AB		A		B		O*	
						4°	37°	4°	37°	4°	37°	4°	37°
1	AB	0	0	0	0	32(8)	0	32(4)	0	64(4)	0	128(8)	0
2	O	++	++	0	0	32(4)	0	16(8)	0	32(8)	8	128(16)	0
3	A	0	++	0	0	256(16)	0	128(8)	0	256(16)	0	256(32)	0
4	O	+++	+++	0	0	1024(64)	0	512(32)	0	512(64)	0	2048(128)	0
5	B	++	0	0	0	1024(64)	0	512(16)	0	512(64)	0	2048(256)	0
6	A	0	++	0	0	512(64)	0	128(16)	0	256(32)	0	1024(64)	0
7	A	0	+++	0	0	512(64)	0	256(16)	0	256(64)	0	512(64)	0
8	O	+++	+++	0	0	256(32)	0	128(8)	0	256(16)	0	512(32)	0
9	O	++	++	0	0	512(64)	0	512(64)	0	512(64)	0	1024(128)	0

* Identical titers were obtained before absorption and are shown for the corresponding sera (Nos. 1 to 9) in Table III. The +++ end-point in the macroscopic agglutination is shown in parentheses along side the + or ± end-points. When not shown, +++ end-point was <4. 0 = <4 (the lowest dilution tested). All sera heated to 56° C. for 30 minutes before this test.

reports concerning cold agglutinins, its independence of the human blood groups was demonstrated. It was of interest to determine whether the same was true for the cold agglutinins found in cases of primary atypical pneumonia.

Experimental. Nine specimens of serum in which the cold agglutinin titers ranged from 128 to 2048 were used in this experiment. These sera were obtained from 9 different cases of atypical pneumonia. In order to avoid hemolysis, the sera were heated at 56° C. for 30 minutes before the tests were carried out. It was found on repeated tests that this amount of heating does not materially affect the cold agglutinin titers. In order to remove the group specific isoagglutinins, group AB cells which had been washed 3 times in saline were used for absorption. The sera were added to equal volumes of the washed and packed AB cells, mixed thoroughly, and incubated at 37° C. for 2 hours with frequent mixing during this period. At the end of this time, the serum was removed after centrifugation and tests for cold agglutinins were carried out with the cells of each of the 4 major blood groups. In the readings, +++ represents a few large tight clumps; + and ± represent the least amounts of definite agglutination readily visible with the naked eye and with the aid of a lens of 3× magnification, respectively. The titers are given as the reciprocal of the final dilution of serum.

The results are shown in Table I. All of the group specific isohemagglutinins had been removed as shown by the results of the microscopic tests which were carried out with the undiluted sera and suspensions of group A and B cells.

Only minor differences were noted in the titers of cold agglutinins obtained with the group O cells and with the cells of the other major blood groups. This was true also for the strong (+++) agglutination which was likewise unaffected. The observed differences are no more than those which are to be expected from cells of different donors, as will be shown later. Furthermore, the titer of cold agglutinins for the group O cells was totally unaffected by the absorption at 37° C.

RELATION OF COLD ISO- AND AUTOHEMAGGLUTININS

A systematic study of this phase was not carried out. During the early part of these investigations, a few specimens of serum and plasma having moderate to high titers were tested simultaneously with autologous cells as well as with group O cells from another donor. Patients of both group O and of other blood groups were included. The cold agglutinin titers were usually found to be identical or showed a 2-fold difference in favor of either type of cells. Similar results were obtained in tests of a few bloods from cases of atypical pneumonia reported by others (4).

Since most of the previous workers had made similar observations with respect to cold agglutinins, these studies were not pursued further, it being assumed that these findings were uni-

formly true. There were 2 cases, however, in which cold autoagglutinins were probably present in moderate or high titers but were not demonstrated at all by the routine tests which employ group O cells of another donor. The cold agglutination was noted in each of these cases in the course of collecting and handling samples of blood for other purposes and in carrying out red blood counts at room temperature. Cold agglutination was noted in the chilled oxalated blood and at room temperature and this was readily dispersed on heating. Sera obtained at the same time and other specimens obtained both earlier and later and tested with group O cells were negative for cold agglutinins in both cases. Unfortunately, quantitative studies of the autoagglutination were not done. It would be of interest to know how frequently this phenomenon occurs. Another worker (7) noted some differences between the cold auto- and cold hetero-agglutinin in the blood of a case of syphilitic cirrhosis of the liver.

COLD AGGLUTININS FOR ERYTHROCYTES OF VARIOUS ANIMALS

To determine the species specificity of the cold agglutinins, tests were done simultaneously with human O cells and with erythrocytes from 9 animal species. The results of such tests in 10 sera without cold isohemagglutinins and in 12 which had them in moderate or high titers are shown in Table II.

All of the animal erythrocytes were agglutinated in the cold to varying titers in the different sera. Rabbit, dog, cat, and rat cells were agglutinated in all of them, guinea pig, sheep, and horse cells in almost all, and hen and monkey cells in more than half of the sera. The highest titers were obtained with the rabbit cells. Dispersal of the cold agglutinated cells at 37° C. also occurred irregularly, more of the agglutination persisting with rabbit than with other cells. With most of the cells, there was no striking difference in the titers obtained in sera with or without cold isohemagglutinins. Agglutination of monkey cells in the

TABLE II

Cold agglutinins for animal erythrocytes in normal human sera and in sera which have cold agglutinins for human "O" cells

Serum	Human "O"		Horse		Rabbit		Guinea pig		Sheep		Dog *		Cat		Rat		Hen		Monkey	
	4° C.	37° C.	4° C.	37° C.	4° C.	37° C.	4° C.	37° C.	4° C.	37° C.	4° C.	37° C.	4° C.	37° C.	4° C.	37° C.	4° C.	37° C.	4° C.	37° C.
1	0	0	20	0	80	10	20	0	20	0	20	0	20	0	80	0	0	0	0	0
2	0	0	40	0	320	40	20	0	80	0	80	0	80	10	40	0	10	0	0	0
3	0	0	160	40	320	40	40	0	40	0	80	40	80	10	80	10	0	0	20	0
4	0	0	80	10	80	10	0	0	0	0	40	0	20	0	40	0	0	0	10	0
5	0	0	10	0	40	10	40	0	10	0	80	0	10	0	80	0	0	0	20	20
6	0	0	20	0	160	10	40	0	10	0	40	0	40	0	40	0	0	0	10	0
7	0	0	20	0	160	10	40	0	20	0	80	10	40	0	160	10	0	0	40	0
8	0	0	40	0	320	10	80	0	20	0	160	10	40	0	160	10	0	0	20	0
9	0	0	0	0	80	20	40	0	10	0	40	0	10	0	80	0	0	0	10	0
10	0	0	80	0	320	20	40	0	0	0	40	0	20	0	20	0	0	0	0	0
11	128	0	0	0	256	32	32	0	8	0	16	0	16	8	32	8				
12	128	0	4	4	512	64	4	0	4	0	64	0	4	0	128	16	16	4	32	0
13	256	0	32	8	128	16	64	4	32	0	32	0	32	4	128	16				
14	2048	0	128	16	256	16	128	32	32	0	32	0	32	16	64	4	8	0	128	4
15	2048	0	32	0	256	32	64	0	64	0	4	0	0	0	8	0	32	0	32	0
16	1024	0	64	0	128	16	128	0	32	0	64	0	16	0	64	4	0	0	256	4
17	512	0	32	0	128	16	128	4	16	0	32	4	32	4	64	8	0	0	512	16
18	512	0	128	4	128	16	128	0	32	0	128	0	16	0	128	4				
19	1024	0	64	16	128	16	128	4	64	0	16	0	16	4	32	8	16	0	128	8
20	1024	0	32	16	128	64	64	4	64	0	128	32	64	8	128	16	32	0	32	0
21	320	0	0	0	320	40	10	0	10	0	80	10	10	0	160	40	0	0	160	160
22	2560	10	80	20	1280	160	320	0	320	0	160	80	160	40	320	80	0	0	1280	80

* Slight to moderate hemolysis in the lower dilutions in all sera.

The sera were all heated at 56° C. for 30 minutes. Lowest serum dilution tested was 1 : 4 in sera 11 to 20 inclusive and 1 : 10 in all others. 0 = negative in the lowest dilution tested, end-point +. Sera 1 to 10 are from normals, 11 to 20 are from convalescent cases of atypical pneumonia, and 21 and 22 from cases of acute hemolytic anemia without pneumonia.

cold occurred more regularly and in higher titers in the sera having cold isoagglutinins and the same was true to less extent with sheep and hen cells. Reversal at 37° C., however, was incomplete with the monkey cells and occurred regularly with the sheep and hen cells.

Serial specimens of serum from several cases of atypical pneumonia were tested at the same time with human and monkey cells. The results in 4 cases are shown in Table III. There was some correlation but no strict parallelism between the cold agglutinin titers with the 2 kinds of cells and considerable agglutination of the monkey cells persisted at 37° C. Similar tests were done with human and sheep cells in serial samples from 5 cases. There was no parallel rise of sheep cell agglutination in 2 of these cases while each of the other 3 showed a 4-fold rise in titer to a maximum of 16 or 32 with complete reversal at 37° C.

An analysis of the cold agglutinins was made only in a preliminary manner by testing the effect of absorption with human O, rabbit, and guinea pig cells in the cold on the titer of cold agglutinins for these and for monkey cells.

Method. Serum in a dilution of 1:10, was mixed with equal volumes of 5 per cent suspensions of cells in saline. The mixture was then kept in an ice bath for 2 hours. At the end of this time, the supernatant fluid was removed after centrifugation in the cold. In order to avoid further dilution, the second and third absorptions were carried out by adding the supernatant fluid to the packed sediment of an equal volume of 5 per cent cells after the saline had been removed. The final absorption was carried out by overnight storage in a refrigerator. The clear supernatant fluid was then used for the cold agglutination tests. The lowest final dilution of absorbed serum, therefore, was 1:20 after the suspensions of the test cells were added.

The results are shown in Table IV. They indicate that rabbit cells contain the broadest cold agglutinating antigen. Absorption with these cells removed the largest amount of cold agglutinin for the human, guinea pig, and monkey cells. Absorption with the human and guinea pig cells, on the other hand, had only slight effect on the titer of cold agglutinins for the heterologous cells.

Other investigators (4) have tested a saline solution of the autoagglutinins of 6 sera from cases of atypical pneumonia with human O and

TABLE III

Results of tests for cold agglutinins carried out with human Group "O" and monkey erythrocytes in 14 sera from 4 cases of atypical pneumonia

Case No.	Day of disease	Human "O"		Rhesus	
		4°	37°	4°	37°
28	10	20	<10	10	<10
	15	1280	<10	320	20
	27	160	<10	160	80
32	6	<10	<10	20	10
	14	<10	<10	80	40
	19	320	<10	160	20
	30	40	<10	80	40
33	13	320	<10	160	20
	14	320	<10	40	40
	25	320	<10	80	20
46	10	<10	<10	40	20
	11	20	<10	40	20
	17	2560	<10	80	20
	31	160	<10	40	20

+ end-points are given.

AB cells and with rabbit, guinea pig, sheep, horse, mouse, and ox cells. In those studies, the titer of cold agglutinins with rabbit cells were essentially the same as with human cells. Guinea pig and pig cells were agglutinated in low titer; horse and ox cells were not agglutinated at all; and only 2 sera were agglutinated in low titer with sheep or mouse cells. In a second experiment, the same authors tested 5 sera with human O, rabbit, mouse, guinea pig, horse, and sheep cells, before and after absorption with autologous cells. In that experiment, moderate titers of agglutinins were obtained with all cells and the highest titers were obtained with rabbit cells. The agglutinins for the animal cells were not removed by absorption of the sera with the autologous cells.

RELATION OF COLD AGGLUTININS TO HEMOLYSINS

The reversible cold hemagglutinin for human O cells was shown to be completely independent of any hemolysin. Several sera which had high titers of cold agglutinins were tested both in the fresh state and after heating at 56° C. for 30 minutes and adding fresh guinea pig serum. No hemolysis occurred with the human O cells, either in the cold or after incubation at 37° C. for 2 hours, in sera from atypical pneumonias or hemolytic anemias.

TABLE IV

Cold agglutinins: Effect of absorption at 4° C. with human "O", rabbit, and guinea pig erythrocytes

Number and diagnosis	Erythrocytes used in adsorptions	Titer of agglutinins at 4° C.							
		Human "O"		Rabbit		Guinea pig		Monkey	
		+++	+ or ±	+++	+ or ±	+++	+ or ±	+++	+ or ±
1 H. A.	None	80	1280	640	512	80	1280	40	640
	Human "O"	<20	<20	20	1280	20	320	20	160
	Rabbit	<20	40	<20	<20	<20	20	<20	40
	Guinea pig	40	640	80	2560	<20	40	<20	160
2 A. P.	None	20	1280	20	2560	<20	320	40	640
	Human "O"	<20	<20	20	320	<20	80	<20	160
	Rabbit	<20	20	<20	<20	<20	80	<20	80
	Guinea pig	<20	160	40	1280	<20	<20	<20	640
3 A. P.	None	160	1280	80	2560				
	Human "O"	<20	<20	40	1280				
	Rabbit	10	160	<20	<20				

H. A. = hemolytic anemia without pneumonia.

A. P. = primary atypical pneumonia.

Varying degrees of hemolysis were noted, however, when fresh sera were tested with cells of each of the animal species except those of the monkey. This hemolysis occurred in the sera of normal individuals as well as in those of the patients. The extent of this hemolysis was independent of the titer of cold agglutinins obtained with the same cells, and its intensity varied with the species from which the cells were obtained. It was most marked and occurred in dilutions up to 1:40 or 1:80 with the cells of the guinea pig, sheep, horse, and dog. It was slightly less marked but occurred quite regularly with hen cells and it was only slight and occurred irregularly with rat cells. With each of these varieties of cells, all of the hemolysis occurred during storage in the refrigerator. Subsequent incubation at 37° C. for 2 hours did not increase the extent of the hemolysis appreciably except in an occasional serum.

When the sera were heated at 56° C. for 30 minutes before the tests, all of the hemolysis was completely abolished except with the dog cells. Slight to moderate hemolysis of dog cells occurred with every serum tested,—those from normals as well as those from the patients,—in the lower dilutions (up to 1:20). This did not usually interfere with the agglutination which occurred in spite of the partial hemolysis and could be read with ease.

SOME PHYSICAL PROPERTIES OF THE COLD AGGLUTININ

Unfortunately, circumstances did not permit extensive physical and chemical studies of the sera containing high titers of cold agglutinins or of preparations of solutions of the cold agglutinin eluted from erythrocytes. Electrophoretic and antibody nitrogen studies have been made (9) on the serum of a case of gangrene of the extremities which had a high titer of cold agglutinins. The agglutinins in that instance were shown to be associated with the gamma globulin and a titer of 2560 at 4° C. was found to be equivalent to 1.473 mgm. of antibody nitrogen. In the course of the present studies, a limited number of observations were made on certain physical properties of the cold agglutinin in its original serum. Among these were the effects of various temperatures, of certain adsorbing agents, and of filtration through various types of filters. Although these studies were not very extensive and were not all adequately controlled, some of the findings are of interest.

EFFECT OF VARIOUS TEMPERATURES

The thermal range of the cold agglutination reaction has been studied by a number of observers some of whom tested sera from cases of atypical pneumonia. In general, the higher the titer, the more likely is the agglutination to per-

sist as the temperature is increased above 0° C. This finding was confirmed in a number of sera early in the course of the present studies. In sera with low or moderate titers, the agglutination was completely abolished at room temperature (22 to 25° C.), while those with high titers in the cold often showed considerable agglutination even at 30 to 35° C. Some of them required incubation up to one-half hour or longer at 37° C. to produce complete dispersion. This was true for sera of atypical pneumonia cases with or without anemia as well as in cases of hemolytic anemia without pneumonia.

The titer of cold agglutinins was not affected by heating at 56° C. for one-half hour in any of the sera tested. Temperatures of 62° C. or higher were necessary in order to reduce the cold agglutinin titers appreciably. At temperatures of 66° C. or higher, all the cold agglutinins were completely removed from the sera. In similar studies made with the blood of cases of trypanosomiasis, it was shown (8) that the cold agglutinins were unaffected by heating for 20 minutes at 58° C. but were completely destroyed at 70° C.

EFFECT OF CERTAIN ADSORBING AGENTS

Method. Each of the adsorbents was passed through a copper sieve having a mesh of 100 micra. The pH of suspensions of these agents in saline ranged between 6.8 and 7.4 and, therefore, no buffers were added in these experiments. Equal volumes of the adsorbent and saline dilutions of serum were mixed thoroughly and incubated

in a water bath of 37° C. for 2 hours with frequent and thorough stirring. The serum dilutions varied from 1:10 to 1:40, the higher ones being used in sera originally having high titers. After incubation, the mixtures were centrifuged at 2900 r.p.m. The clear supernatant fluid was then removed and tested for cold agglutinins in the usual manner. Sera of patients with hemolytic anemia or atypical pneumonia that had moderate to high titers of cold agglutinins were used in these tests.

The effect of the different adsorbents varied considerably. A list of the agents used and a summary of their effect on the cold agglutinin titers is given in Table V. Casein, silicon dioxide, alundum, and wood charcoal had no discernible effect on the titers in any of the sera that were tested. Bone charcoal, blood charcoal, and norit reduced the titers of most of the sera but left some unaffected. Vegetable charcoal and permutit reduced the titers in all of the sera tested but to a varying extent. Fuller's earth and kaolin removed all of the cold agglutinins completely or nearly so.

EFFECT OF FILTRATION

The result of the preceding experiment suggested the possibility that filtration through certain types of filters might materially reduce the cold agglutinin titers. A number of tests were carried out with various filters including new as well as previously used ones of some types. Divergent results were obtained with the various filters as shown in Table VI.

Filter paper and fritted glass filters of fine

TABLE V

Effect of certain adsorbing agents on the cold agglutinin titers of sera from cases of hemolytic anemia and of primary atypical pneumonia

Adsorbing agent	Number of sera		Effect on cold agglutinin titers
	H.A.	A.P.	
Casein	3	6	None
Silicon dioxide	3	5	None
Alundum	1	3	None
Wood charcoal	3	6	None
Bone charcoal	2	5	2 H. A. unaffected, others reduced 4 to 16-fold.
Vegetable charcoal	2	5	All reduced 4 to 32-fold.
Blood charcoal	4	5	2 A. P. unaffected, others reduced 8-fold or more.
Norit	4	5	3 (2 A. P. and 1 H. A.) unaffected, others reduced 16-fold or more.
Permutit	3	4	2 (1 A. P. and 1 H. A.) reduced 4-fold, others reduced 16-fold or more.
Fuller's Earth	4	5	All completely removed or nearly so.
Kaolin	4	5	All completely removed or nearly so.

H. A. = hemolytic anemia cases; A. P. = cases of primary atypical pneumonia. The original titers of these sera before adsorption ranged from 320 to 5120. The smallest final dilution tested after adsorption was 20 in most instances; and 40 and 80 in the case of 2 of the sera with the highest original titers.

TABLE VI
Effect of filtration on cold agglutinin titers

Serum	Diagnosis	Titer before filtration	Titer after filtration								Fritted glass	Seitz EK ₃
			Filter paper	Mandler		Berkefeld filters						
				Used	New	"W" used	"W" new	"N" used	"V" used	"V" new		
1	A. P.	640	640									<10
2	H. A.	640	640	640	640				80	320		<40
3	H. A.	640			80		<40			320		<40
4	H. A.	5120										320*
5	A. P.	1280			320		40			640		640
6	A. P.	1280								640†	1280	<20
7	A. P.	640	640	<20			<20			<20	640	<10
8	A. P.	160				40		<20	<20			<10
9	A. P.	1280							<160			<160
10	A. P.	5120							<160			<160
11	A. P.	80		<20								
12	A. P.	320				<20	<20		<20			

H. A. = hemolytic anemia without atypical pneumonia.

A. P. = atypical pneumonia. Serum No. 10 was from a case of A. P. after severe hemolytic anemia occurred.

Ten layers of Whatman Nos. 2, 5, 42, and 50 were used in small Seitz filter holder. The Mandler filter was of "average" porosity. The fritted glass was 12G4 (filter size).

* Titer 640 obtained with this serum when the experiment was repeated.

† Same titer obtained when experiment was repeated.

porosity had no effect. Variable results were obtained with Mandler filters and Berkefeld filters of different porosities but the used ones removed some or all of the agglutinins more often than did the new ones. Seitz filters, on the other hand, removed the agglutinins from 8 of 10 sera tested and materially reduced the titer in 1 of the others. Attempts to elute the agglutinins from the filter pads were not successful.

One of the objects of these experiments was to see whether cold agglutinins in hemolytic anemia could be differentiated from those in atypical pneumonia. These studies failed to accomplish that aim.

EXPERIMENTS WITH PNEUMONIC LUNG AND OTHER TISSUES

Preliminary experiments were made on the relation of the affected lung from fatal cases of atypical pneumonia to the cold agglutination reaction. In one experiment, suspensions of the diseased lung obtained at autopsy from 6 patients in whom antemortem sera showed moderate titers of cold isohemagglutinins were tested for cold agglutinins. The tissues were incubated at 37° C. for an hour and then thoroughly ground with alundum after adding 4 volumes of saline. After an additional 30 minutes of incubation, the mix-

tures were centrifuged and the supernatant fluids, treated like serum, were tested for cold agglutinins with human O cells. No agglutination occurred with any of these preparations.

In a second experiment, the effect of such lung preparations on the cold agglutinins of the patients' sera was tested. Antemortem sera from 2 cases were set up for the cold agglutination test in triplicate. One of the sets of tubes containing 0.5 ml. each of serial dilutions of serum and equal volumes of 2 per cent suspension of human O cells was used as a control. To each tube in the second and third sets were added 0.1 ml. amounts of a 1:5 and 1:50 dilution, respectively, of the patients' own cleared lung suspension. Agglutination was then read after storage in the ice box over night. In one of these tests, the cold agglutinin titer was reduced from 160 to 10 by the concentrated suspension of lung and to 40 by the 1:50 suspension. In the second test, the control titer was only 20 and no agglutination occurred after addition of either of the lung suspensions.

Two other sera from recovered cases were tested in a similar manner with uninvolved and with consolidated portions of lung and with spleen obtained at autopsy from cases of bacterial pneumonia, and also with normal mouse lung. One of these sera had a cold agglutinin titer of 640

and the titer of that serum after addition of 20 per cent tissue suspensions ranged from 40 to 80. In the second serum, the original cold agglutinin titer was 160 and the titers of the same serum with 20 per cent tissue extracts added in 0.1 ml. amounts was 40 to 80. In neither case was there any difference between the effect of spleen or of normal or infected lungs.

Hemoglobin solutions and suspensions of red blood cell stroma were used in the same manner as the tissue suspensions. In these experiments, the concentrations of hemoglobin and of red blood cell stroma suspensions were based on the volumes of packed red blood cells, and the concentrations added to the serum-cell mixtures in the cold agglutination tests corresponded to those used in the experiments with other tissues. Neither the hemoglobin solutions nor the suspensions of red cell stroma had any effect whatever on the cold agglutinin titers of the same sera.

These findings suggest the possibility that the affected lung from cases of atypical pneumonia could remove or inhibit the cold agglutinins to a greater extent than the same or other tissues from other sources. Further observations, however, are necessary in order to determine the validity of these findings.

DISCUSSION

This paper has been devoted to a presentation of a number of observations on the cold agglutinins found in cases of primary atypical pneumonia and of hemolytic anemia. While the results of these studies have some general interest merely as biological observations, the objective of the studies was to define, if possible, the nature of the cold agglutinins in primary atypical pneumonia with the idea of shedding some light on the etiology of this disease. The results of most of these studies revealed no differences between the cold agglutinins in cases of atypical pneumonia and those in the cases of hemolytic anemia without pneumonia. The possibility that the two conditions might be related, however, has some basis. This is suggested by the occurrence of hemolytic anemia, as well as other phenomena such as thrombophlebitis and acrocyanosis, in occasional cases of atypical pneumonia. Cases of these conditions without pneumonia have been found to

be associated with autohemagglutination. The relationship, however, may be quite indirect and similar to the finding of heterophile antibodies as a result of a large variety of bacterial infections or following immunizations with bacteria or foreign proteins. The common antigen has not been identified. The present studies indicate quite definitely that the cold agglutinin is not a Forssman antibody though it may be some other type of heterogenetic antibody.

Some of these results, though preliminary in nature, are presented because of their general interest. It is possible that further studies by others who are in a better position to pursue them may yield useful information and shed more light on the now obscure nature of the cold agglutination phenomenon and also on the nature and causes of primary atypical pneumonia and of some of the other conditions associated with cold agglutinins.

SUMMARY AND CONCLUSIONS

A number of serological and physiological properties of cold agglutinins were studied in sera obtained from a number of cases of primary atypical pneumonia and from 2 cases of hemolytic anemia without pneumonia.

Cold agglutinin titers are the same in serum and plasma obtained from the same blood.

The complete independence of cold agglutinins and blood group isohemagglutinins was confirmed. Absorption of the group-specific agglutinins from sera containing moderate or high titers of cold agglutinins left the latter intact.

While cold autohemagglutination and cold isohemagglutination almost always co-exist, suggestive evidence was obtained in two cases of the possible independence of these two reactions.

Cold agglutinins for the erythrocytes of several animal species were found both in normal sera which lacked cold agglutinins for human O cells and in sera from cases of atypical pneumonia and hemolytic anemia in which cold agglutinins for human O cells were found in high titer. The erythrocytes of some of these animals were agglutinated more strongly and in higher titer in sera containing high titers of cold agglutinins for human cells than they were in the sera of normal individuals. Rabbit cells showed the strongest

cold agglutination and, in absorption tests, appeared to have the widest antigenic action. Some rises in titer of cold agglutinins for sheep and monkey cells were observed in a few cases of atypical pneumonia but the reversal of the agglutination with the monkey cells at 37° C. was irregular and incomplete.

The cold isohemagglutinin was not related to any hemolysin demonstrable *in vitro* for either human or animal cells.

Cold isohemagglutination is dispersed as the temperature is raised above 5° C. The thermal range in any given blood depends on the titer and intensity of the cold agglutination reaction in that blood. In most instances, the agglutination is completely dispersed at room temperature (20 to 25° C.) but in sera of high titer, incubation at 37° C. for 30 minutes or longer may be required to give complete dispersion.

The cold isohemagglutinins are unaffected by heating at 56° C. for 30 minutes. Partial destruction of the cold agglutinin occurs at 62° to 65° C. and the agglutinins are completely destroyed by heating for 30 minutes at temperatures of 66° C. or higher.

Certain common adsorbing agents had no effect whatever on the cold isohemagglutinins while others removed them either partially or completely from some or from all of the sera tested.

Filtration through common types of bacterial filters at room temperature may remove some or all of the cold isohemagglutinins. Seitz asbestos filter pads were most active in removing the cold isohemagglutinins.

There was no difference in the behavior of sera from cases of atypical pneumonia and of hemolytic anemia without pneumonia with respect to the effects of heating, of adsorbing agents, or of

filtration. The same was true of the agglutination of animal erythrocytes.

Preliminary observations on the effect of various tissues suggest the possibility that the diseased lung of cases of atypical pneumonia may inhibit the cold isohemagglutinin to a greater extent than do tissues from other sources. These studies, however, need further extension and confirmation.

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COLD AGGLUTININS. IV. CRITICAL ANALYSIS OF CERTAIN ASPECTS OF THE METHOD FOR DETERMINING COLD ISOHEMAGGLUTININS

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The data recorded in the previous papers of this series are based upon tests carried out in a more or less uniform manner. The method of testing for cold agglutinins (1) is essentially the one which has been found (2) most suitable in the studies of blood from cases of hemolytic anemia. It was readily reproducible when the same materials were employed in repeated tests carried out at the same time. When, however, the same sera were retested on other occasions, slight, and sometimes considerable, differences in titer were noted. For that reason, as these investigations progressed, batches of sera were subjected to comparative tests, using the routine method and some variation of this method. The present paper is devoted to a critical evaluation of certain details of the quantitative technique for determining cold agglutinins in human serum as revealed by these comparative tests. Most of the data were accumulated during the course of the present investigations, but some were the results of separate experiments.

EFFECT OF AGE OF CELLS ON COLD ISOAGGLUTININ TITERS

It was noted early in the course of these studies that some sera in which cold agglutinins could not be demonstrated with cells that were used on the day the blood was drawn nevertheless showed significant titers when tested with the same cells after they had been allowed to stand 3 or 4 days. It seemed important, therefore, to determine how often and to what extent this phenomenon occurred. A number of sera were, therefore, tested simultaneously with cells obtained on different days from the same donor.

Tests of cold agglutinins were usually carried out 2 or 3 times each week throughout this study. On a number of these occasions, batches of sera, both positive and nega-

tive, were tested at the same time with fresh or 1-day-old cells and with other cells from the same donor that had been used for previous tests and were 3 or 4 days old at the time. The cells had remained suspended in saline during this period. They were kept in a refrigerator when not in use and were discarded if hemolysis occurred. A single set of dilutions was made and distributed in duplicate tubes in order to minimize errors from this source. In these tests and in the succeeding ones, all of the agglutinations were observed at 4° C. and were completely reversed at 37° C.

The accumulated results of the comparative tests are shown in Table I. One-fourth of the sera showed the same titers with the fresher and with the older cells, but almost twice as many showed higher titers with the older cells. When compared separately, lower titers were more frequent with fresh than with 1-day-old cells and higher titers were somewhat more frequent with 4-day-old cells than with 3-day-old cells. An appreciable number of sera which gave no agglutination in a dilution of 1:10 (the lowest dilution tested) when fresh or 1-day-old cells were used, yielded titers ranging from 10 to 320 with 3- or 4-day-old cells. A similar number of positive sera showed titers which were 8 or more times greater with the older than with the fresher cells. These observations indicate that 3- or 4-day-old cells are appreciably more sensitive to agglutination in the cold than are fresh or 1-day-old cells except in sera having a high content of cold agglutinins.

COMPARISON OF RESULTS WITH CELLS FROM DIFFERENT DONORS

In the preceding comparisons, the cells used in the tests of each serum were obtained from the same donor. It was not feasible to use a single donor throughout all of the studies. It was of interest, therefore, to obtain information on the extent to which variations in the titer of cold ag-

TABLE I

Comparison of cold isohemagglutinin titers (end-point +) of the same sera tested simultaneously with fresh or 1-day-old cells and with 3 or 4-day-old cells of the same donor

Titer with fresh or 1-day-old cells	Titer with 3 or 4-day-old cells											Total
	<10	10	20	40	80	160	320	640	1280	2560	5120	
<10		2	5	8	5	2	2					24
10			3	4	2							9
20	1			9	8	3	1					22
40	3	2	2	11	13	10	5	2	1			49
80	1		1	2	7	25	7	2	1	1		47
160			1	4	6	24	11	9				55
320					7	22	33	26	3	4		95
640						15	18	13	1	2		49
1280							5	5				10
2560										1		1
5120											1	1
Total	5	4	12	38	48	101	82	57	6	8	1	362

SUMMARY

Same titer with fresh or 1-day cells and with 3 or 4-day-old cells..... 90 (25 per cent)
Higher titer with 3 or 4-day-old cells..... 177 (49 per cent)

<10 with fresh or 1-day cells, 10 or higher with 3 or 4-day-old cells..... 24
 2-fold..... 88
 4-fold..... 43
 8-fold..... 16
 16 or 32-fold..... 6

Higher titers with fresh or 1-day old cells..... 95 (26 per cent)

<10 with 3 or 4-day cells, 10 or higher with fresh or 1-day cells..... 5
 2-fold..... 55
 4-fold..... 34
 8-fold..... 1

Note: The 95 sera in which the titer with fresh or 1-day cells was 320 were about equally divided among those having the same (35 per cent), higher (30 per cent), and lower (35 per cent) titers with the older cells.

Among the 206 sera in which the titer with the fresher cells was less than 320, 69 per cent had higher titers with 3 or 4-day cells; whereas, 70 per cent of the 61 sera in which the titer with the fresh or 1-day cells was greater than 320 had lower titers with the old cells.

glutinins occurred when cells of the same age but obtained from different donors were used in tests of the same sera. Two series of experiments were carried out for this purpose; the cells of two donors were used in the first and those of 8 donors were tested in the second.

In the first experiment, blood was obtained at the same time from two donors (F and S). The bloods were washed 3 times and 2 per cent suspensions were made and stored at 5° C. The procedure was repeated 4 days later. Sera previously found to have low or moderate titers were tested at the same time with these 4 cell suspensions. In the second experiment, erythrocyte suspensions from 8 donors were all used in tests of the same sera on the day the cells were obtained and on 5 or 6 successive days thereafter. As in the previous tests, a single series of dilutions was made of each serum and distributed into separate rows of tubes in order to minimize variations resulting from this source. In order to indicate the intensity of the reaction, both the ++ and the + end-points are recorded.

The first of these experiments was done with 27 sera. Considerable variations in the sharpness of the end-point were obtained in different sera as indicated by the discrepancies between the ++ and the + end-points. This was true for both the fresh and 4-day-old cells of each donor. A comparison of the titers obtained with the cells of the 2 donors indicated that the cells of S were, in general, more sensitive to agglutination in the cold and thus gave higher titers than did the cells of F. The differences, however, were greater with the fresh cells, and with them the ++ end-points obtained with the cells from both donors varied somewhat less than did the + end-points.

Thus, the fresh cells of donor S showed ++ agglutinations in dilutions of 10 to 40 in 5 sera in which there was no agglutination with the cells of F in the lowest dilution tested, namely 1:10. In the remaining 22 sera, the ++ end-point with fresh F and S cells was the same in 18 sera and only twice as high with the cells of the one

or the other donor in the 4 remaining sera. The + end-points on the other hand were higher more often with the cells of donor S. The same + end-point was obtained with the cells of the 2 donors in only 8 sera, including 1 in which the titer was less than 10. In all but 1 of the 19 remaining sera, the titers with S cells were 2- or 4-fold greater than with F cells and in 3 of them, titers of 20 or 40 were obtained with S cells and no agglutination was obtained with F cells. When 4-day-old cells were used, on the other hand, there was much less difference between the cells of the 2 donors. Thus, ++ agglutination was obtained in the same titer with both F and S cells in 12 sera and higher titers were obtained in 9 sera with the former and 6 with the latter. The final titers (+) were the same with the 2 donors' cells in 18 sera, and the remaining 9 showed only 2-fold differences in titer, favoring the F cells in 5 instances and the S cells in 4.

The cells of both donors behaved alike with respect to the effect of the age of the cells. Both gave higher titers with the 4-day cells than with the fresh cells. The intensity of the cold agglutination with both donors' cells, as indicated by the ++ end-points, was correspondingly greater with the 4-day-old cells. In this respect, however, greater differences were noted with the cells of F than with the cells of S.

In summary, therefore, the cells of both F and S gave higher titers with 4-day-old cells than with fresh cells. These differences were more marked with the cells of F. With fresh cells, higher titers were obtained more often with the cells of S. With the 4-day-old cells on the other hand, there was essentially no difference in the results obtained with cells of the 2 donors.

In the second experiment, 6 sera of high titer from cases of atypical pneumonia were employed on 2 occasions. Here again the greatest differences were noted with the fresh cells. In general, the titers increased through the second or third day and then declined. The greatest decline, however, occurred when 5- or 6-day-old cells were used, the titers with these cells being comparable to those obtained with fresh cells. In only 1 of the 6 sera were the titers obtained with fresh cells the same or higher than with older cells. In that serum, the titers began to decline after the second day. The variations in the titers obtained with the cells of different donors were less striking than those obtained with the cells of different age ob-

TABLE II

Comparison of cold agglutinin titers obtained with Group O erythrocytes of different ages and from various donors

Serum	Donor of cells	Fresh		1 Day		2 Days		3 Days		4 Days		5 and 6 Days *	
		2+	1+	2+	1+	2+	1+	2+	1+	2+	1+	2+	1+
1	A	20	80	80	160	80	80	320	640	160	320	80	320
	B	20	40	80	320	160	1280	320	1280	80	320	80	320
	C	20	20	80	160	80	640	320	320	160	640	80	80
	D	20	80	160	320	160	1280	320	1280	160	320	80	160
	E	20	40	160	320	320	1280	640	1280	80	320	80	320
	F	20	80	160	640	160	320	160	2560	80	320	20	40
	G	20	20	160	320	320	640	320	2560	80	320	40	80
	H	20	40	80	320	320	1280	160	640	80	320	80	160
2	E	320	640	160	160	320	320	160	160	160	160	40	40
	G	640	1280	160	320	320	640	160	320	80	320	80	80
	H	160	640	160	320	320	640	160	320	320	320	40	80
	I	320	640	160	640	160	1280	160	160	80	160	80	80
	J	160	640	160	320	320	640	160	160	80	320	40	40
	K	160	640	160	640	160	320	80	160	160	320	80	80
	L	320	640	160	320	320	320	160	160	160	320	80	160
	M	320	640	320	320	320	2560	320	640	320	640	80	160
3	E	40	40	320	640	160	320	320	640	160	640	80	160
	G	40	40	320	640	640	2560	320	320	160	320	40	80
	H	40	40	320	1280	320	320	320	320	160	640	80	80
	I	40	40	160	1280	160	640	320	640	160	640	40	80
	J	40	40	320	640	320	320	320	640	160	320	80	80
	K	40	40	320	640	320	320	160	320	160	160	80	160
	L	40	40	640	640	320	320	160	320	160	320	80	160
	M	40	40	640	1280	320	1280	320	640	320	320	40	80

* Serum 1 was tested with 5-day-old cells and sera 2 and 3 with 6-day-old cells. They were 1 to 10 weeks old at the time of these tests.

When no agglutination occurred beyond the ++ end-point, the same titer is listed under +.

tained from the same donor. The results in 3 of the sera, which illustrate most of the variations observed, are shown in Table II.

Thus, it would appear from the results of these two experiments that the age of the cells is an important factor in the cold agglutinin titer. This is true both in sera of low and of high titer but the most marked differences are noted in sera having low titers. Considerable variations in titer are obtained with cells of the same age obtained from different donors, particularly when the sera are of low or moderate titer.

COMPARISON OF TITERS WITH 2 PER CENT AND WITH 0.2 PER CENT CELLS

In the routine test for cold agglutinins, equal volumes of 2 per cent cells were added to the serum dilutions. This concentration of cells gives a fairly heavy suspension. In sera of high or moderate titers, the readings are easily made because of the large clumps which are formed. It

was considered possible, however, that agglutination of lesser intensity might be obscured because of the density of this suspension. To examine this possibility, tests were carried out simultaneously with 0.2 per cent suspensions of cells.

In these tests, the 0.2 per cent suspension was made by further dilution of the 2 per cent cells used in the same tests, 3-day-old cells from several donors being used. Batches of sera were tested simultaneously with both concentrations of cells. As before, a single series of dilutions of the serum was prepared and divided between 2 rows of tubes. A scale of reading was adapted for the tests with 0.2 per cent cells which corresponded to that used with 2 per cent cells.

The results of 400 simultaneous tests with these 2 concentrations of cells are shown in Table III. Among the 172 positive tests, slightly more than one-third yielded similar titers with both concentrations of cells. In the remaining tests, higher titers were obtained 3 times as often with 0.2 per cent cells as with 2 per cent suspensions. The

TABLE III

Cold isohemagglutinins. Comparison of titers (1+) obtained with 2 per cent and with 0.2 per cent suspensions of the same cells tested simultaneously in the same sera

Titer with 2 per cent cells	Titer with 0.2 per cent cells									Totals
	<10	10	20	40	80	160	320	640	1280	
<10	228	9	6	2	3					248
10	6	3	8	1	1					19
20	1	1	6	6	11	3				28
40	0	2	1	10	11	2				26
80	1				17	8	1	1		28
160	1				4	16	5			26
320					1	1	5	2		9
640						1	3	5	2	11
1280							2	2	1	5
Totals	237	15	21	19	48	31	16	10	3	400

Comparison of results with 2 per cent and 0.2 per cent cells	No.	Per cent	Corrected * per cent
Same titers.....	291	72.8	36.6
Titers higher with 0.2 per cent cells.....	82	20.5	47.7
2 per cent = <10; 0.2 per cent = 10 or higher.....	20	5.0	11.6
2-fold higher.....	42	10.5	24.4
4-fold higher.....	15	3.8	8.7
8-fold higher.....	5	1.3	2.9
Titers higher with 2 per cent cells.....	27	6.8	15.7
0.2 per cent = <10; 2 per cent = 10 or higher.....	9	2.3	5.2
2-fold higher.....	12	3.0	7.0
4-fold higher.....	6	1.5	3.5
Totals.....	400	100	100

* By exclusion of the 228 tests in which the titer was <10 with both 2 per cent and 0.2 per cent cells. The final concentration of cells in the mixtures were $\frac{1}{2}$ of the stated amounts whereas the titers are given as the final dilution of the serum after the cells are added.

differences, however, were not very great and occurred mostly in sera of low titer.¹

RESULTS WITH POOLED ERYTHROCYTES FROM SEVERAL DONORS

One intensive experiment was carried out with 67 sera and a pool of erythrocytes derived from 8 group O donors. The sera varied widely in their cold agglutinin titers and were several months old at the time. One-half of the pooled blood was stored in the original citrated plasma and the cells of the remainder were washed and made up as a 2 per cent suspension in saline. The sera were tested simultaneously with 4 concentrations of fresh cells, namely 2.0, 1.0, 0.5, and 0.2 per cent. On 6 successive days thereafter, the sera were retested with similar concentrations of cells in duplicate, one set of suspensions being freshly made up each day from the original stored citrated blood and the other from the cells which had been stored as a 2 per cent suspension in saline.

The results corroborated those of the previous experiments with respect to the age of the cells. The strongest agglutination and the highest titers were obtained with cells 2 to 4 days old, the optimum varying in different sera. With fresher and with older cells, the agglutinations were progressively weaker and the titers were lower. The concentration of the cells had much less effect. The end-points were easiest to read when the 1.0 per cent suspension was used than with the higher or lower concentrations. The titers, however, varied less than in the previous experiment in which 2.0 and 0.2 per cent suspensions were compared. The cells which were stored in the original plasma gave higher titers and clearer end-points than the cells which were stored as a 2 per cent suspension in saline.

SHARPNESS OF THE END-POINT

The simplest quantitative measure of the intensity of the cold agglutination reaction was the titer to which strong agglutination was noted. The difference between the highest concentration of serum giving strong agglutination and that at which the weakest definite agglutination is discerned can be taken as a measure of the sharpness

TABLE IV
Cold isohemagglutinin titers. Comparison of ++ and + end-points

Titer ++	Titer +	2.0 per cent cells		0.2 per cent cells	
		Sera	Percent	Sera	Percent
<10	10	55	4.1	11	6.9
	20	74	5.5	16	10.0
	40	60	4.5	7	4.4
	80	23	1.7	6	3.8
	160	5	0.4	1	0.6
	320	2	0.1	1	0.6
	Total	219	16.3	42	26.3
10 or more	Same as ++	169	12.6	37	23.1
	2-fold	615	45.8	56	35.0
	4-fold	280	20.8	18	11.3
	8-fold	55	4.1	7	4.4
	16-fold	6	0.4	0	0
	Total	1125	83.7	118	73.8
Total positive sera		1344	100.0	160	100.0

The tests with 2 per cent cells include those in which the erythrocytes were of different ages up to 4 days but the 0.2 per cent cells used in these tests were 3 days old.

of the end-point. Since there were many sera of comparatively low titer, the ++ end-point was chosen to indicate the strong reaction. The + end-point was used for the final titers since it seemed more likely, thereby, to exclude non-specific reactions which were seen as slight agglutination (\pm), visible only with magnification.

The differences between the ++ and + end-points are summarized in Table IV. Among the sera which failed to show ++ agglutination, the + end-points ranged up to 320. This represents differences ranging up to 64-fold, provided that ++ agglutination were present at all in dilution below 10. In most of these sera, however, the + end-point was between 10 and 80. The sera which showed stronger cold agglutination as indicated by ++ agglutination in titers of 10 or higher gave appreciably sharper end-points. Only rarely was there a 16-fold difference between the ++ and + end-point, and in all but a few sera, there was a 4-fold difference or less. No definite or uniform relation was discernible between the sharpness of the end-point and the age and concentration of the cells.

From these observations it may be said that sharp end-points were associated with high titers and with strong agglutination, but in general, the

¹ Similar observations were reported at the recent meeting of the Society of American Bacteriologists (3).

end-points were fairly distinct considering the character of the reaction.

DISCUSSION

The test for cold agglutinins in human serum as usually performed, and as carried out routinely in the studies that were reported in this series of papers, is a very simple one to perform. In sera of high titer, it is very easy to read and to interpret. In sera of low titer, however, both the reading and the interpretation may not be so simple. Many factors may influence the quantitative results of these tests. In some sera, particularly those of low titer, the cold agglutinins may not be revealed by the test under certain circumstances. If the test for cold agglutinins is to be used as a diagnostic aid,—and its value in this respect was indicated by the data presented in the earlier papers of this series,—an acquaintance with the pitfalls and possible errors involved in the test is essential. It is for that reason that the present studies were undertaken.

Only a few of the technical features of the method as they affect the results of the test were analyzed in this paper. Others were considered in the preceding paper of this series, and the effect of storage of the sera on the titers of cold-agglutinins will be considered in the paper which follows. These studies were not intended to include all of the technical details or to indicate accurately the statistical errors involved in each phase of the technique. Only some of the more important features which should be kept in mind in determining cold isohemagglutinin titers in human sera were considered.

The data presented indicate that the age of the cells that are used in the test for cold agglutinins is an important factor in determining the intensity of the cold agglutination as well as the titer, particularly in sera of low or moderate cold agglutinin content. In such sera, the use of fresh or even 1-day-old cells may fail to reveal cold agglutinins which can be demonstrated in moderate titers when 2- to 4-day-old cells are used. Furthermore, the differences in the sensitivity of cells from different donors is appreciably lessened when the older cells are used.

Cells of lower concentration than the one routinely used may also yield higher titers in some

sera in which the cold agglutinin content is low. This may be due to the fact that the greater dispersion of the cells offers a better opportunity to observe minor degrees of agglutination. However, with concentrations as low as 0.2 per cent, the reading of the test is more difficult, particularly near the end-points, and 1.0 per cent suspensions are more satisfactory in this respect.

The sharpness of the end-point is, in a way, a measure of the accuracy of the quantitative measurements. In this respect, there did not seem to be any uniform differences related to the age of the cells or to their source. Here, again, the cold agglutinin content was more important, sharper end-points being obtained in sera of high titer.

From the point of view of the patient and his disease, these factors may not be of great significance. If the cold agglutinating property of serum during the course of an infection or early in convalescence is an important factor in bringing about such complications as hemolytic anemia, thrombophlebitis, acrocyanosis, etc., it is obviously only the patient's own circulating cells and plasma which are involved. The conditions to which the blood is subjected in various parts of the body and its content of cold autohemagglutinins are probably the only factors which are of importance. Of these, only a measure of the autohemagglutinin content is obtainable in the test tube; its relation to isohemagglutinins was considered in the preceding paper.

SUMMARY AND CONCLUSIONS

The results of comparative tests for cold isohemagglutinins carried out simultaneously with cells of different age, from different donors, and in different concentrations were presented. Considerable variations in the intensity of the agglutination and in the titers were obtained in individual sera under these conditions. The greatest variations were obtained in sera in which the cold agglutinin content was low. In such sera, the use of cells which were 2 to 4 days old gave significantly higher titers with greater regularity than did fresh or 1-day-old cells. With the 2- to 4-day-old cells, moreover, the differences between the titers obtained with the use of cells from different donors were minimized. Cells more than 4 days

old behaved in these respects more like fresh cells. In comparative tests, in which 2 per cent and 0.2 per cent cells were used, the latter yielded higher titers more frequently than the former. A concentration of 1.0 per cent, however, proved more satisfactory technically. Cells stored in the original plasma were more satisfactory than those which were stored in saline.

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COLD AGGLUTININS. V. DETERIORATION OF COLD ISOHEMAGGLUTININS ON STORAGE

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When the first instances of marked cold hemagglutination were noted in this laboratory in cases of primary atypical pneumonia of unknown etiology (1), a number of sera were available from other similar cases which had been studied during the preceding months. Most of these sera had been obtained from cases in this hospital but many were from other hospitals. The large majority of them gave negative results when tested for cold agglutinins. On the other hand, sera from characteristic cases obtained at the proper time in the course of the disease from patients who were being currently observed gave positive results.

Several possible explanations for this discrepancy were considered. In the first place, it was possible that the earlier cases differed from the current ones with respect to the occurrence of cold agglutinins. This explanation seemed unlikely in view of the similarity of the clinical course in both groups of cases. It seemed possible, however, that variations in severity of the cases might have accounted for the differences. The earliest cases in which the cold agglutinins were observed were very severe ones and many of the others that were being encountered at that time seemed to be of greater severity than those which had been described from schools and from military hospitals. Many of the sera which had been stored were from mild cases in young adults and some of them were from military hospitals. Interestingly enough, most of the few positive results among the stored sera were from very severe cases in which there was extensive pulmonary involvement (2).

Another possible explanation depended on the likelihood that most of the sera that were stored had been improperly prepared and preserved. It is usually more convenient to collect a number of specimens of blood, store them in a refrigerator over night, and take off the serum on the following day. The possibility must, therefore, be considered that, without special precautions having

been taken to warm the bloods, some at least of the cold agglutinins might have been adsorbed on the red blood cells of the clot and left behind in the process of taking off the serum. Such a loss can readily be demonstrated, particularly if the blood remains cold while the serum is rapidly removed. For the most part, however, the bloods were kept at room temperature for some time before the sera were separated. Subsequent observations have indicated that under the latter circumstances very little drop in the titer of cold agglutinins occurs.

Finally, the possibility was considered that the cold agglutinins had deteriorated on storage. This was suggested by other workers (3) to explain the low titers of some of the stored sera from characteristic cases of atypical pneumonia in their series. In another study (4), more than one-third of the sera had been stored for several months although it is not stated what proportion of the negative sera in cases of atypical pneumonia were among those which had been stored. Alternative explanations were given by the latter authors for most of the negative results which they obtained in their cases of primary atypical pneumonia.

The present paper deals with a study which was directed specifically towards determining the effect on the titer of cold isohemagglutinins of storage for varying periods up to 18 months in an ordinary electric refrigerator, and for shorter periods at room temperature.

MATERIALS AND METHODS

Almost all of the sera in this study were obtained at various stages of the disease from the patients with characteristic primary atypical pneumonia of unknown etiology, who were included in the previous study (2). A few sera which were obtained from 3 of the patients with acute hemolytic anemia (5) are also included.

The bloods were all obtained and handled with sterile precautions. In many instances, the sera were separated at room temperature (20 to 25° C.) but most of them were warmed to 37° C. before the serum was taken off.

Special care was taken to obtain serum free from cells. The sera were then stored in sterile rubber stoppered test tubes in an ordinary household refrigerator in which the temperature varied between 5 and 10° C. Many of these sera were also used for a variety of other tests which were carried out at the same or at different times. These sera were allowed to come to room temperature before each of the tests was carried out and were often kept at that temperature for several hours while the tests were being set up. Toward the end of this study, due to unavoidable circumstances, all of the sera were kept at room temperature for 3 to 6 weeks. The final tests were done after this period.

The method used for determining the titer of cold agglutinins was described in a previous paper (5). The results recorded in the present study are based upon tests carried out with a single method. Briefly, the tests were set up as follows: to 0.5 ml. of serial 2-fold dilutions of serum were added equal volumes of a 2 per cent suspension of 2- to 4-day-old group O red blood cells from a single donor. The mixture was shaken and kept in a refrigerator over night. The tests were then read, usually after the racks were placed in an ice bath. The agglutinations in all positive tests were completely dispersed at 37° C. The initial tests on each of these sera were carried out either on the day when the blood was obtained or within 3 or 4 days. The tests were repeated on large batches of sera at irregular intervals, either in connection with other tests of the same sera or when otherwise convenient. The titers are expressed as the reciprocal of the greatest final dilution of serum that gave definite (1+), coarsely granular, or floccular agglutination which was visible without magnification.

RESULTS

A total of 753 tests were done on the 248 sera, or an average of 3 tests of each serum. The original titers varied from 10 to 5120. In 19 of the sera, one additional test was done after the titer had dropped below 10. The results obtained in the last test of these sera are not included in the analysis since they only confirmed the results of the previous one. Excluding these 19, there were 486 repeat test of the 248 sera and these formed the basis of the analysis which follows.

The outstanding feature of the results of the repeated tests was the irregularity and unpredictability of the rate of deterioration of the cold agglutinin titers. To be sure, some of the variations may be due to the different conditions to which some of the sera might have been subjected inadvertently. Many of these variations are not measurable since, in a general way, all of the sera were handled in the same manner.

In a few specimens, contaminants were en-

countered, particularly in the form of molds or putrifying bacteria. The variations from the previous levels in such sera were not strikingly different from those observed in sera which had remained sterile throughout. In fact, some of the most heavily contaminated ones retained their original titers for several months after the contaminants first appeared and without special treatment of the serum in the interim. In others, however, there were varying degrees of hemolysis of the red blood cells which resulted from the contaminants and which obscured the end-points.

Considering the number of variables involved and the difficulty of measuring most of them, the total number of sera tested is obviously inadequate for the purpose of defining accurately the rate of deterioration of the cold agglutinin titers in relation to all of the factors involved. One feature which seemed to be important in determining the persistence of the cold agglutinins was the intensity of the original reaction and this was, in general, proportional to the titer of the serum. The results are, therefore, considered in relation to the titers obtained in the initial and subsequent tests. Unfortunately, circumstances did not permit complete studies of all of the sera at the same intervals or at frequent and regular intervals.

A few points require comment. In the first place, some of the sera were exhausted earlier than others. In addition, some of them lost their agglutinating properties earlier than others. The numbers of sera in which this took place naturally accumulated with time and the deteriorated sera were not retested after the titer dropped below 10, except as already mentioned. The accumulated results of the later tests, therefore, appear to denote less deterioration than was actually the case.

Titers higher than the original ones were obtained in a number of sera that were retested at varying intervals even after 16 months. Most of these higher titers were observed between 7 and 15 months after the initial tests. Two possible explanations may be offered for these results. Evaporation may conceivably account for the concentration of the antibodies in some of the sera. Few, if any of the sera, however, showed sufficient evaporation to account for the observed 2- or 4-fold increases in titer on the basis of that factor alone. Another plausible explanation for the higher titers depends upon the errors involved

in the tests. The higher titers in the later tests were obtained mostly in sera which initially had the lowest titers and the frequency with which these apparent increases were observed diminished steadily as the original titers increased. Thus, about one-third of the tests which were repeated on sera which initially had titers of 10 or 20 showed 2- or 4-fold higher titers:—about 1 in 4 of the sera with initial titers of 40; 1 in 6 of those which originally had a titer of 80; and about 1 in 10 of those with original titers of 160 or 320 had higher titers when retested. Among the sera in which the original titer was 640 or higher, none was found to have a greater titer when subsequently retested. This correlation seems to be quite regular and probably is significant. It may depend, in part at least, on the fact that the endpoints were more sharply defined in sera of high titers and less well defined in those of low titers so that errors in reading the original and subsequent titers might be limited mainly to the sera of low titer. Some of these discrepancies may also depend on the use of cells from different donors.

No other uniform or obvious trend could be discerned. A study of the individual sera in which 3 or more tests were done in the course of this study likewise revealed no uniformity. In some of these sera, progressively lower titers were obtained in each successive test. In others, there was an early drop in the titers which then remained stationary and occasionally even showed apparent increases of 2 or 4-fold over the results of the preceding tests. Still other sera showed

higher titers in the second or third tests, or in both, and subsequent ones showed a drop in titer.

The same variations were also noted with respect to the changes in titer resulting from exposure of the sera to room temperature. In general, a larger proportion of the latter tests showed a drop in titer, and the reductions were, on the whole, considerably greater than those observed over much longer periods in the same sera before they had been exposed to room temperatures or in other sera which had not been exposed to the higher temperatures. Nevertheless, the same general changes were noted, that is, some sera showed marked drops, others only moderate drops in titer, and still others showed no change or an apparent increase in titer in spite of the exposure to room temperature.

These wide variations and the relatively small number of observations have made it difficult to define the pattern of the deterioration of the cold agglutinin titers. A few correlations were made and while they are of some interest, most of them are not very revealing. In Table I, there are listed the numbers of sera showing changes of various extent from the original cold agglutinin titers, arranged according to the time elapsed after they were first tested. The outstanding feature revealed in this table is the relatively large proportion of the older sera which showed large decreases in titer or loss of cold agglutinins after exposure to room temperature. No striking progression of the deterioration can be discerned from these results. In this table, of course, the height of the original titer is not taken into consideration.

TABLE I
Deterioration of cold isohemagglutinins on storage

Change from initial titer	Months after initial determination					Totals
	4 or less	5 to 6	7 to 9	10 to 15	16 or more	
2 or 4-fold increase	2	3(2)	25(4)	23(1)	9(9)	62(16)
No change	5	8	40(7)	31	7(7)	91(14)
2-fold decrease	5	11(3)	37(7)	21(2)	18(18)	92(30)
4-fold decrease	6	6(1)	20(4)	21(2)	12(12)	65(19)
8-fold decrease	4	7(2)	10(1)	12(2)	18(18)	51(23)
16-fold or greater decrease	1	5	7(1)	12(6)	13(13)	38(20)
Decrease to <10 *	2	11(8)	17(7)	30(16)	27(27)	87(58)
Totals	25	51(16)	156(31)	150(29)	104(104)	486(180)

* Not included above.

Numbers in parentheses refer to sera which had been kept at room temperature (20 to 25° C.) for 3 to 6 weeks prior to testing.

TABLE II
Time of disappearance of cold isohemagglutinins in relation to original titer

Initial titer	Months after initial determination when titer first decreased to <10						No. of sera tested	Percentage of sera decreased to <10
	4 or less	5 to 6	7 to 9	10 to 15	16 or more	Total		
10		2(1)		3(1)	2(2)	7(4)	11	64
20	1	2(2)	5(2)	3(2)	5(5)	16(11)	30	53
40	1	2(1)	3	9(4)	5(5)	20(10)	33	61
80		3(2)	4(2)	4(1)	2(2)	13(7)	35	37
160		2(2)	2(2)	7(4)	5(5)	16(13)	46	35
320			1	3(3)	8(8)	12(11)	55	22
640			2(1)	1(1)		3(2)	19	16
1280 to 5120						0	19	0
Total <10	2	11(8)	17(7)	30(16)	27(27)	87(58)	248	35
Number of sera tested	25	51(16)	156(31)	150(29)	104(104)	248*		
Percentage of sera decreased to <10	8	19(50)	11(23)	20(55)	26(26)	35		

Numbers in parentheses refer to sera which had been kept at room temperature (20 to 25° C.) for 3 to 6 weeks prior to testing.

* The apparent discrepancy between this total and the sum of the numbers to the left of it in the same horizontal line is due to the fact that many sera are listed in more than one of the vertical columns.

The time of essential disappearance of the cold agglutinating properties of the sera is correlated with the original titers in Table II. As the original titer increased, there was a steady drop in the proportion of sera in which the titer was observed to drop below 10. This was, of course, to be expected. Thus, all of the sera in which the original titer was 1280 or higher retained some of the cold agglutinins throughout the period of study, whereas more than one-half of the sera in which the original titer was 40 or less, had dropped to a level below 10 in the same period and an intermediate number of those with original titers of 80 to 640 had such a drop.

The proportion of all the cases in which a reduction in titer occurred showed no uniform trend in relation to the length of time they were stored. This is due in part to the irregular proportion of cases of different titers which were examined at the different intervals. Furthermore, no allowance is made in this or in the preceding table for sera in which the titers had previously dropped below 10 and therefore were not included in the subsequent tests. Their inclusion would, of course, tend to increase the proportion of sera showing deterioration at successive intervals.

An attempt was made to find an expression for the deterioration in titers of cold agglutinins that would include most of the important factors in-

involved. It was necessary to take into account the original titers, the wide variation in the rate of deterioration from these titers that occurred in different sera, and the fact that changes in titer occurred in geometric proportions. Any expression of the changes should tend to minimize the observed increases and give proportionately greater weight to the larger variations. If the original titer is represented as 5×2^n and subsequent titers are indicated as 5×2^m , the ratio of the sums of the exponents m and n would satisfy most of these qualifications. The ratio would represent an average and provide a fair approximation of the proportion of the original titers which persist. In order to express the extent of deterioration, this ratio readily may be converted to express the percentage drop from the original titer by applying the following formula:

$$\text{Index of deterioration } (D) = \left(1 - \frac{\sum m}{\sum n}\right) \times 100.$$

This index has been calculated for all the sera tested at various intervals and also for the results of all of the tests done on sera which originally had the same titer. The results are shown in Table III. The effect of the exposure to room temperature is also shown in this table and was obtained by calculating separately the index of deterioration in those sera which had been so exposed.

TABLE III

Estimation of the deterioration of cold isohemagglutinin titers

According to:	Index of deterioration *		
	A. All sera	B. Sera stored 3 to 6 weeks at 20 to 25° C.	C. All sera excluding those listed in B
Months of storage:			
4 or less	29		29
5 to 6	34	54	28
7 to 9	21	29	19
10 to 15	33	67	24
16 or more	49	49	
Original titers:			
10	15	100	-38†
20	20	53	-10†
40	41	53	35
80	36	50	25
160	33	54	23
320	29	47	19
640	32	38	29
1280	33	45	25
2560	37	46	32
5120	32	44	25
Total—All sera	32	48	23

* Index of deterioration (D) is calculated according to the formula:

$$D = \left(1 - \frac{\sum m}{\sum n} \right) \times 100$$

the original titer of the serum having the value 5×2^n and each of the values subsequently observed being given the value 5×2^m . When the titer is <10 , m is given a value of 0 for present purposes.

† Titers were higher than the original ones.

Note: The table showing all of the data upon which these numbers are based has been omitted to conserve space.

From this table, it is seen first of all that deterioration in the cold agglutinin titers was greatly accelerated by exposure to room temperature. Roughly speaking, there was approximately the same degree of deterioration during the 3 to 6 weeks when the sera were exposed to room temperature as took place during the entire preceding period of several months when the sera were kept in the refrigerator. This is also evident from the percentages shown at the foot of the columns in Table II.

From the manner in which these data were obtained, it was not possible to demonstrate the progression of the deterioration with time. This has already been alluded to previously. The index of deterioration, as shown in Table III, does not appear to be greater after different periods of storage except as this was influenced by the ex-

posure to room temperature. When, however, account is taken of the progressive number of sera which had deteriorated to a titer below 10 at each successive interval, the index would tend to increase with time as might be expected. The effect of the height of the original titer on the deterioration is also indicated in Table III. Among the groups of sera which originally had titers of 10 or 20, there appears to be an increase rather than a decrease in titer. This is probably more apparent than real. The number of observations is small and, as already mentioned, the greatest errors are probably involved in the titrations of the sera which have such low titers. In all other sera, the index of deterioration was essentially the same and seemed to be independent of the original titer.

DISCUSSION

From the data presented, it is seen first of all that appreciable deterioration of cold agglutinin titers takes place with the usual type of storage of sera in an ordinary electric refrigerator over a period of several months. No uniform rate of deterioration was noted in these sera and wide variations occurred. In sera which had low or moderate titers, a large proportion retained their original titers or even showed apparent increases. Such increases over the original were not observed among the sera which originally had very high titers. Decreases in titer of varying extent were observed among each of the various groups of sera that were tested.

The results of the tests in sera exposed for a relatively brief period at room temperature showed similar variation. The deterioration, however, was more marked and occurred more regularly when compared with the changes observed after the longer periods of storage at 5 to 10° C.

It is to be borne in mind that the sera on which this study is based were originally collected specifically for the purpose of determining cold agglutinin titers. Precautions were taken to avoid adsorption of agglutinins from these sera before they were separated and stored. Some data were obtained which indicated that some reduction in titer may take place if care is not taken to separate the serum at temperatures ranging between 20 and 37° C. Only slight reductions in titer occasionally occur in sera of low or moderate titer

which are separated at room temperature instead of at 37° C. Greater or significant losses can be demonstrated only in sera of very high titers (6, 7).

Some evidence has also been obtained to indicate that the original cold agglutinin titers may be completely preserved by lyophilizing the serum. Through the courtesy of Dr. T. Hale Ham, a serum obtained from a patient with acute hemolytic anemia in 1939 was tested for cold agglutinins after it had been lyophilized and kept at room temperature for 4½ years. The titer at the end of this period was identical with that obtained before lyophilization. After the serum was reconstituted, it deteriorated to a moderate extent during several months of storage in a refrigerator and markedly after it had been left at room temperature.

It is not unlikely that the negative results of the tests for cold agglutinins in the sera collected from patients with primary atypical pneumonia during the 1941-42 season and tested more than a year later were the result of a combination of factors. These include: (1) the failure to observe proper precautions in order to avoid the loss of cold agglutinins during the collection and separation of the sera, (2) deterioration of the cold agglutinins on storage, and (3) the original low titers of the sera. The latter, in turn, may have been due to the fact that most of the cases from which those sera were obtained were relatively mild (2). These factors must be taken into account in evaluating the quantitative results based on stored sera.

Because of the wide variations observed and the relatively small number of sera and tests, it has been difficult to express the extent and rate of deterioration accurately. The proposed index of deterioration is an arbitrary expression intended to convey roughly the amount of drop in titer in terms of a percentage of the original titer. It is, necessarily, only a rough approximation of the amount of deterioration and takes into account several significant factors but not all of them. For example, there is no allowance made in this formula for variations in the accuracy of the test. It will, however, serve to point up the need for some simple method of measuring and expressing the deterioration in the biologic properties of sera.

For more accurate estimation of the rates of deterioration, it would be desirable to have large numbers of sera and test them all at the same regular intervals. Clinical material is difficult to obtain and to handle in such a manner.

According to the formula presented, there is essentially the same degree of deterioration in all sera, irrespective of their original titers. Obviously, cold agglutinin titers below some arbitrary one, such as 10 or 20, that is considered to be the lowest significant titer, will be reached at different times depending on the original titer. Likewise, among the sera of any given age, the proportions which deteriorated to a level below the significant titer will increase in inverse proportion to the height of the original titer. In the present study, unfortunately, the same sera are not represented in each of the intervals so that such a progression is not indicated.

SUMMARY AND CONCLUSIONS

The titer of cold isohemagglutinins was determined in 248 sera at the time they were obtained and at irregular intervals during the ensuing 18 months. The original titers of these sera ranged from 10 to 5120. The sera were stored throughout this period in an ordinary electric refrigerator which had a temperature range from 5 to 10° C. Many of them were kept at room temperature (20 to 25° C.) during the last 3 to 6 weeks before the final tests were done.

A large proportion of the sera showed significant deterioration in their cold isohemagglutinin titers after storage at refrigerator temperature for several months. The rate and extent of the deterioration varied markedly in different sera. Many of those which originally had low or moderate titers retained those titers unchanged or showed an apparent increase even after more than a year of storage. Others showed decreases in titer of varying extent and at different intervals.

Deterioration of cold agglutinin titers occurred with greater regularity and at a much more rapid rate after the sera were allowed to remain at room temperature.

The method of collecting sera and the factor of deterioration must be considered in the interpretation of the results of tests for cold agglutinins that are done on stored sera.

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COLD AGGLUTININS. VI. AGGLUTININS FOR AN INDIFFERENT STREPTOCOCCUS IN PRIMARY ATYPICAL PNEUMONIA AND IN OTHER CONDITIONS AND THEIR RELATION TO COLD ISOHEMAGGLUTININS

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The isolation of an indifferent streptococcus (Number 344) from the lung of a fatal case of primary atypical pneumonia has recently been reported (1) and its biological properties described. Convalescent sera from more than one-half of the cases of atypical pneumonia which were tested agglutinated this strain in final dilutions ranging from 1:10 to 1:160 whereas agglutination in low titers occurred only rarely in the acute phase sera of such cases, in sera of normal individuals, or in acute or convalescent sera from persons with other respiratory diseases. The results of the streptococcal agglutination tests in atypical pneumonia correlated in many instances with the results of cold hemagglutination tests and with complement fixation tests with mouse lung antigen (2). In a number of cases, however, positive results were obtained with only one or two of these tests. Similar strains were also isolated from other cases.

The authors did not feel that their evidence warranted the conclusion that the bacterium is a factor in the etiology of primary atypical pneumonia. Nevertheless, the fact that the positive findings were restricted largely to cases of atypical pneumonia is of some interest. The present paper deals with the results of serological tests in cases of atypical pneumonia and of other conditions using streptococcus 344 and another similar strain (E. S.) isolated in this laboratory. Tests for cold hemagglutinins were also done in all of the sera included in this study.

MATERIALS AND METHODS

A total of 255 sera from 78 cases of atypical pneumonia and 330 sera from 163 other individuals were used in this study. The latter include serial specimens from 6 cases of rheumatic fever and 82 individual sera from non-rheumatic carriers and non-carriers of hemolytic streptococci. All of the rheumatic patients and a number of the others

had recent streptococcal infections. The antistreptolysin titers of these sera were known. The sera and the antistreptolysin data were generously provided by Drs. T. Duckett Jones and Benedict F. Massell, of the House of the Good Samaritan, who had carried out repeated throat cultures in these cases. Antistreptolysin determinations were also carried out in their laboratory on serial specimens from a number of the cases of atypical pneumonia. The sera in the atypical pneumonias were tested after they had been stored from 3 to 15 months. All but a few of the remaining sera were tested within a few weeks of the time they were obtained.

Streptococcal agglutinations were carried out in essentially the manner described by Thomas *et al.* (1). A transplant of strain 344 was obtained from Dr. Thomas and strain E. S. was isolated in December 1943 from a sputum obtained late in the disease in a characteristic and severe case of atypical pneumonia. It was found in almost pure culture in this sputum and it had cultural, biological, and serological properties similar to those of strain 344. The antigens were prepared by washing the sediment of broth cultures 3 times in saline and then resuspending it in saline in one-third of the original volume of the culture. The suspensions were then heated at 65° C. for one-half hour and stored at 5° C. when not in use. For the tests, equal volumes of the antigens and of serial dilutions of serum, beginning 1:5, were incubated for 18 hours at 37° C. before reading. The positive sera gave floccular agglutinations similar to those obtained with pneumococci. Some of the control sera gave a finely granular type of agglutination in the lowest dilutions and these were visible only with the aid of a magnifying lens. The titers were read as the reciprocal of the highest final dilution of serum giving floccular agglutination. The method used in the cold hemagglutination tests is described in a previous paper (3).

RESULTS

The number of cases in each of the various groups studied and the maximum titers of agglutinins obtained in the cases of each group, both with strain 344 and with strain E.S., are shown in Table I. It is seen at a glance that the maximum titers in cases of primary atypical pneumonia differed from those found in each of the other

groups. Titers of 40 or higher were obtained in about one-half of the cases of atypical pneumonia when strain 344 was used and in about three-fourths of the cases when strain E. S. was used. Such titers were obtained in only 5 per cent or less of the cases in the other groups with either of the two strains. In general, the agglutination which occurred in the sera of cases other than atypical pneumonia were qualitatively less marked than were the agglutinations that occurred in similar dilutions of sera from cases of atypical pneumonia. Furthermore, the development of agglutinins for the indifferent streptococci and the occurrence of rises in titer or of increases in the intensity of the agglutination during the course of the disease or in convalescence was limited almost entirely to the cases of atypical pneumonia.

Before considering the results in the cases of atypical pneumonia a few of the relevant features of some of the other cases of interest, particularly those in which the maximum titers of streptococcal agglutinins was 40 or higher, will be considered briefly.

In one of the normal subjects who did not have any respiratory infection and was not a carrier of hemolytic streptococcus, the titer of agglutinins was 320 for strain

344 and 1280 for strain E. S. These agglutinations were obtained in a single specimen of serum which was cloudy; the agglutinations were fluffy in all of the dilutions and the substrates were not clear as in other sera in which strong agglutination occurred. There were no cold hemagglutinins in this serum.

One of the cases listed among the non-respiratory infections was a case of fever of undetermined origin that lasted many weeks and failed to respond to sulfonamides, penicillin, salicylates, and non-specific therapy. The titers obtained in several sera in this case were all 40 with strain 344 and 20 or 40 with strain E. S. There were no cold agglutinins in any of this patient's sera.

Four of the cases of bacterial pneumonia are of interest. In one of them, a titer of 20 was obtained with both of the strains during the fourth and fifth week of the disease. Serum obtained during the seventh week gave a titer of 80 with strain 344 and 40 with strain E. S. This patient had type 7 pneumococcus pneumonia and a recurrent and prolonged bacteremia probably due to bacterial endocarditis. He recovered following intensive penicillin therapy. There were no cold agglutinins in any of his sera. In a second case, the titer with strain 344 rose from 20 to 80 and that for E. S. rose from 40 to 160. This patient had a diffuse bronchopneumonia of both lower lobes; type 12 pneumococcus was obtained from the sputum; blood cultures were negative and the leukocyte count was 4,850 during the acute stage of the disease and rose to 14,000 later. He showed a very slow response to treatment with penicillin and may have had an underlying atypical pneumonia of non-bacterial etiology. Cold agglutinins, how-

TABLE I
Occurrence of agglutinins for two strains of indifferent streptococci in cases of primary atypical pneumonia associated with cold agglutinins and in various other conditions

Diagnosis	Maximum agglutinin titer										Total number of cases
	Streptococcus 344					Streptococcus E.S.					
	<10	10	20	40	80 or higher	<10	10	20	40	80 or higher	
Primary atypical pneumonia, all cases	7	11	23	15	22	1	8	11	15	43	78
Cold agglutinins: 20 to 80	3	5	5	4	6	1	1	6	3	12	23
(Maximum titers) 160 to 320	3	5	11	4	7	0	6	3	4	17	30
640 or higher	1	1	7	7	9	0	1	2	8	14	25
Hemolytic streptococcal infections	6	3	1			7	2	1			10
Carriers without infection	17	13	3			21	9	3			33
Acute rheumatic fever	3	3				4	1	1			6
Subacute bacterial endocarditis	3	1	1	1		3	1	1	1		6
Bacterial pneumonia	17	5	5		2	15	4	6	3	1	29
Influenza A	11	1	1			11	1	1			13
Other respiratory infections	6	1				6		1			7
Non respiratory infections	11	3	1	1		9	6			1	16
Normal (non-strep. carriers)	15	15	8		1	19	11	8		1	39
Hemolytic anemia (no pneumonia) (cold agglutinins 40 to 2560)	4					4					4
Total—not atypical pneumonia	93	45	20	2	3	99	35	22	4	3	163

ever, were not obtained in any of the sera in this case. In a third case, streptococcal agglutinins were not demonstrated during the acute phase and titers of 20 and 40 were obtained with strains 344 and E. S., respectively, early in convalescence. The patient had diffuse bronchopneumonia; type 18A pneumococci were obtained in the sputum and blood cultures were negative. The illness in this case was complicated by cirrhosis of the liver, pulmonary tuberculosis, nitrogen retention in the blood, and congestive heart failure. The response of the pneumonia to penicillin was slow but the patient eventually recovered. In this case, the cold agglutinin titer was 20 both early and late in the disease. In the fourth case, the titer of streptococcal agglutinins obtained with each of the two strains during the acute phase was 10 and the titers ~~obtained~~ in convalescence were 20 and 40 with strains 344 and E. S., respectively. This patient had typical lobar pneumonia; types 7 and 23 pneumococci were obtained from the sputum early in the disease; blood cultures were negative and the patient showed a good response to penicillin therapy. There were no cold agglutinins in any of his sera.

One of the cases of influenza is of particular interest. In this case, the illness began at the end of February 1944 and an influenza virus was isolated from throat washings obtained early in the course of the disease but this virus could not be identified as influenza A or B. This was the only case of clinical influenza among a large number studied during that season (4) in which cold agglutinins developed. Cold hemagglutinins could not be demonstrated in the first two sera obtained in this case during the acute phase of the disease but they appeared during convalescence and reached a maximum titer of 80. Weak agglutination for both strains of indifferent streptococci was obtained in a titer of 40 in the acute phase sera and stronger agglutination occurred in the convalescent sera in the same titer with strain E. S. and in a titer of 80 with strain 344. There were a few râles heard in the lungs of this patient during the febrile period but x-rays were negative. It is possible that this represents a mild case of primary atypical pneumonia.

One additional case is of interest because of the occurrence of cold agglutinins. In this case, a cold hemagglutinin titer of 160 was obtained both in acute and convalescent sera but agglutinins were not demonstrated either for strain 344 or for strain E. S. This was a case of lobar pneumonia in which type 7 pneumococci were obtained from the sputum, blood cultures were negative, and the patient responded favorably to penicillin therapy. In this case, however, there was a history of severe cough of 3 weeks' duration prior to the abrupt onset of the symptoms of lobar pneumonia. This was the only case of bacterial pneumonia in which cold agglutinins were demonstrated in any significant titer.

CORRELATION OF STREPTOCOCCAL AND COLD HEMAGGLUTININ TITERS

There was some correlation, though not a very close one, between the maximum titers of cold

isohemagglutinins and the maximum titers of agglutinins for the two strains of indifferent streptococci. The correlation was somewhat better with strain E.S. than with strain 344. In individual sera, there were wide discrepancies between the titers of cold agglutinins and of the streptococcal agglutinins. There were some instances in which relatively high titers of streptococcal agglutinins were noted in sera with only low or moderate titers of cold agglutinins and others in which low titers of streptococcal agglutinins were obtained in sera which had cold agglutinins in high titer. The titers obtained with the two strains of indifferent streptococcus, however, were in much closer agreement. In general, the titers were higher in the cases of atypical pneumonia with strain E.S. than with strain 344. On the other hand, agglutinins with strain E.S. were observed more frequently during the acute stage of the disease. In the sera of cases other than atypical pneumonia, the titers obtained with the two strains were more nearly identical. A better idea of the relation of the cold agglutinin titers and the titers for strains 344 and E.S. to each other and to the course of the disease in cases of atypical pneumonia may be had from the data shown in Table II.

Cross absorption tests were done in a few of the sera which showed both cold hemagglutinins and agglutinins for the indifferent streptococci. The results were uniform and corresponded to those reported by others (1). Absorption at 37° C. with strain 344 completely removed the agglutinins for this strain and for E.S. but left the cold hemagglutinin titers intact. The same was true when strain E.S. was used. Absorption in the cold with human O cells removed the cold hemagglutinins but left the agglutinins for the two strains of streptococci unaffected.

RELATION OF AGGLUTININS FOR INDIFFERENT STREPTOCOCCI TO HEMOLYTIC STREPTO- COCCAL INFECTIONS AND TO THE ANTI- STREPTOLYSIN TITER OF THE SERUM

Through the courtesy of Dr. T. Duckett Jones, many of the sera used in the present study were tested for their content of antistreptolysin. There was little difference in the streptococcal agglutinin titers obtained in the cases of rheumatic fever

TABLE II

Comparison of cold isohemagglutinin titers and titers of agglutinins for two strains of indifferent streptococci in selected cases of primary atypical pneumonia of unknown etiology

No.	Days after onset	Agglutinin titers			First day afebrile	No.	Days after onset	Agglutinin titers			First day afebrile	
		Cold (Iso-)	Strep. 344	Strep. E.S.				Cold (Iso-)	Strep. 344	Strep. E.S.		
1	9	0	20	80	19	6	8	0	0	40	10	
	13	10	80	320			10	20	80	80		
	18	1280	160	320			14	160	160	160		
	21	160	80	320			28	20	160	160		
2	9	20	0	10	14	7	8	0	10	10	17	
	13	320	0	10			11	20	10	40		
	14	320	20	40			15	640	40	160		
	16	640	20	40			19	320	20	80		
	19	320	20	80			24	320	20	20		
	21	40	80	80			29	40	40	40		
	87	10	0	0			48	0	20	20		
3	7	0	10	20	10	8	11	0	0	0	13	
	11	20	20	40			16	40	0	10		
	13	40	20	80			18	80	10	20		
4	10	40	0	20	20		23	160	20	40		16
	11	80	0	20			29	20	20	40		
	13	320	20	20	9	11	640	80	160	21		
	15	320	20	40		19	1280	40	160			
	19	320	20	80		46	10	10	20			
	21	160	40	160	10	13	0	0	20	26		
	24	640	40	80		21	320	20	40			
	27	160	40	40		28	320	40	320			
	29	320	20	40	11	12	320	40	20	26		
	33	320	20	40		19	2560	160	160			
	64	160	10	0		26	5120	40	160			
5	10	0	0	20	20*	12	29	640	10	0	26	
	17	10	40	160			9	0	0	0		
	21	40	20	160			16	40	0	0		
	24	0	20	40			26	640	40	40		

* Relapse of fever on day 21, died of hemolytic streptococcal superinfection on the 24th day.

In this table, titer 0 = <10 (the lowest dilution tested).

See also Figures 4 to 7—in paper II of this series for additional cases.

and in the non-rheumatic subjects, irrespective of whether or not they were carriers of hemolytic streptococci. Furthermore, there were no differences in titers of agglutinins for each of the strains of indifferent streptococci in sera having a high titer of antistreptolysin and in those with low titers of the latter. In the cases of atypical pneumonia, as in those of other diseases not associated with hemolytic streptococci, no significant titers or rises in titers of antistreptolysin occurred. This was true both in sera in which agglutinins for the indifferent streptococci developed and in those in which such agglutinins failed to appear. A few isolated sera obtained in various stages of the disease showed unusually high titers of antistreptolysin. In almost every instance, these sera

were heavily contaminated, usually with putrifying bacteria.

RESULTS IN CASES OF HEMOLYTIC ANEMIA

In a previous paper of this series (5), sera of moderate or high cold hemagglutinin titer were subjected to a variety of adsorbing agents and to filtration through various types of bacterial filters. Some of these procedures removed the cold agglutinins from the sera, either entirely or in part. Some of these sera were obtained from cases of atypical pneumonia and others from cases of hemolytic anemia. No differences were discerned in the behaviour of the sera obtained from these two types of cases with respect to the effect of these procedures. In the present study are included

sera from 4 cases of hemolytic anemia in which the cold agglutinin titers ranged from 40 to 2560. All of these sera failed to show any agglutinins for either of the two strains of indifferent streptococci. These findings suggest that agglutination with these streptococci may serve as an aid in differentiating two types of hemolytic anemia, one resulting from atypical pneumonia and the other independent of that disease. This differentiation is not an absolute one, however, since there were a small number of cases of atypical pneumonia in which the cold hemagglutinin titers ranged from 160 to 2560 and agglutinins for both strain 344 and E. S. were absent or occurred only in a titer of 10.

DISCUSSION

The serological findings previously reported (1) have been corroborated for the most part. As compared with these observations, however, agglutination with strain 344 occurred with greater frequency in the acute phase of atypical pneumonia, in normal individuals and in the cases of other infections included in the present study. Among the cases of atypical pneumonia, a significantly greater proportion had titers of 10 or 20 than were found in other conditions. Titers of 40 or higher were limited mostly to the sera from cases of atypical pneumonia. Moreover, the development of agglutinins for streptococcus 344 in the course of the disease and the occurrence of significant rises in titer were also limited almost entirely to the cases of atypical pneumonia. The results of the tests in which strain E. S. was used were, in general, parallel to those obtained with strain 344. The former was agglutinated in higher titers in many of the cases of atypical pneumonia. With strain E. S., however, agglutination occurred more often in the acute phase sera of some of the atypical pneumonias. In sera from cases other than atypical pneumonia, the agglutinations obtained with the two strains were more nearly alike.

Strain E. S. was obtained with ease because it occurred in almost pure culture and was recognized and identified from a routine culture made from sputum on the surface of a blood agar plate. No systematic attempt was made to isolate strains of indifferent streptococci from other cases of

atypical pneumonia or of other conditions. Histological studies of the lungs in fatal cases, however, failed to reveal any evidence that such an organism plays any part in the disease.

Several features of the data presented are worth mentioning. In the first place, the sera in the cases of atypical pneumonia had all been stored for several months before the streptococcal agglutination tests were carried out. Sera from the remaining cases on the other hand, were either fresh or only a few weeks old when they were tested. The cold hemagglutinin titers had definitely deteriorated and to a considerable extent in a larger proportion of these sera (6). It is possible that the streptococcal agglutinins had also deteriorated to some extent in the serum of the cases of atypical pneumonia. If that is so, then the titers of agglutinins for the indifferent streptococci in the cases of atypical pneumonia may have been higher than those recorded. The differences between the titers obtained in atypical pneumonias and in other conditions would then be correspondingly greater.

On the other hand, the cases of atypical pneumonia are not entirely representative.¹ They include only cases in which cold agglutinins were present. Sera from cases in which cold agglutinins did not develop were unfortunately not available for this study. The occurrence of agglutinins for indifferent streptococci in characteristic cases of atypical pneumonia in which cold hemagglutinins do not develop cannot be stated from this study. The results of the cold agglutinin studies previously reported (3) and the correlations of the streptococcal and the cold hemagglutinin titers suggest that agglutination with the indifferent streptococci would probably develop less frequently and in lower titers in cases of atypical pneumonia in which cold hemagglutinins do not develop. The significance of the present findings, like those of the cold agglutinins, is a matter for speculation. The results reported in this paper, as well as those reported by others (1), indicate some relationship between these indifferent streptococci and primary atypical pneumonia but, as

¹ Characteristic cases of primary atypical pneumonia were encountered only rarely during the winter of 1943-44. Only stored sera were, therefore, available from such cases for this study.

those authors have pointed out, they cannot be interpreted as indicating an etiological rôle for these streptococci. The findings, however, do suggest a possible antigenic relationship between the causative agent of primary atypical pneumonia, of these indifferent streptococci and of human erythrocytes. The results of the absorption tests suggest further that the antigen which these streptococci have in common with the unknown agent of atypical pneumonia is different from that which the latter shares with human red blood cells. It is possible, however, that the indifferent streptococcus coexists as a non-pathogen which becomes antigenic in atypical pneumonia.

One point which has emerged from the present studies is of interest and may have a limited significance from a hematological point of view. Cases may be encountered in which hemolytic anemia is discovered but the antecedent history is difficult to interpret. The present findings, though they are based on a limited number of cases, suggest that agglutination with strains of indifferent streptococci similar to 344 may serve as an aid in differential diagnosis. Agglutination of these strains in the serum of such cases would strongly suggest atypical pneumonia as the predisposing factor or antecedent illness in such a case. Failure to demonstrate such agglutinins would tend to rule out this possibility, but will not definitely exclude it.

SUMMARY AND CONCLUSION

Sera obtained from 78 cases of atypical pneumonia and from 163 other persons, including normal subjects and patients with various types of streptococcal disease and of other respiratory infections, were tested for agglutination with the indifferent streptococcus 344 and with a similar strain isolated from the sputum of a case of atypical pneumonia. In conditions other than atypical pneumonia, maximum titers of 10 or 20 were obtained with either strain in about one-third of the cases and higher titers were obtained in only 5 per cent or less. In cases of primary atypical pneumonia, on the other hand, only a few failed

to show agglutination with these strains, while one-half of the patients developed maximum titers of 40 or higher for strain 344 and three-fourths of the patients developed agglutinins in such titers for strain E. S.

There was some correlation, though not a very close one, between the maximum cold isohemagglutinin titers and the maximum titers of agglutinins obtained with the two strains of indifferent streptococci. Cross absorption tests indicate that these two kinds of agglutinins are unrelated.

There was no correlation whatever between the titers of cold hemagglutinins or of the agglutinins for the indifferent streptococci and the antistreptolysin titers of the sera. This was true both in the cases of atypical pneumonia and in those of hemolytic streptococcal infections.

The agglutination of indifferent streptococci similar to those used in this study may serve as an aid in the differential diagnosis of certain cases of hemolytic anemia.

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STUDIES ON PAIN: QUANTITATIVE MEASUREMENTS OF TWO PAIN SENSATIONS OF THE SKIN, WITH REFERENCE TO THE NATURE OF THE "HYPERALGESIA OF PERIPHERAL NEURITIS"

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The study of sensation in intact human skin has revealed that noxious stimulation results in the perception of pricking and burning pain (1, 2). Furthermore, patients with peripheral neuropathy often experience burning pain on contact with the bed clothes or with slight manual pressure, and yet report an impaired perception of pricking pain. There is disagreement as to whether these are entirely different qualities of pain, or rather aspects of the same quality, seemingly different because of time factors. An attempt has been made with quantitative methods to understand the significance of these two pain sensations of the skin.

METHOD

The technique which utilizes radiant heat as a source of painful stimulation (3) was used for measuring the threshold for both "pricking" and "burning" pain. Observations were made of the amount of thermal radiation just necessary to produce the sensation of pain. The light from a 1000 watt bulb was focussed on an area of blackened skin 3.5 cm.² in area. Intensity of stimulus was measured in gram cal. per sec. per cm.². An automatic shutter allowed the light to fall on the skin area for a measured period of time. At the end of a trial, the instructed subject reported the sensation. If no pain was experienced, the intensity was increased and the procedure repeated at 60-second intervals, until the subject just perceived pain at the end of the exposure. The amount of energy necessary to produce pain was measured by a radiometer, and several measurements agreeing within ± 5 per cent were taken to establish the threshold.

Each subject was carefully instructed as to the purpose and method of the investigation. For the "pricking" pain threshold there was clearly described the sensation of warmth mounting into heat which would suddenly, just at the end of a given exposure, "draw together" into a sharp prick or tingle. It was also demonstrated that the pain threshold or the "edge of pain" which the subject was asked to report would be similar to the sensation perceived on striking a pin point lightly against the skin. For the "burning" pain threshold, the sensation of warmth

was described as mounting into heat which would turn not quite so suddenly, but nevertheless definitely, into a burning pain which could best be perceived at the end of a 5- to 10-second exposure. This "burning" pain is localized readily in the area stimulated. There is no punctate discreteness, but rather a blunt or slightly diffuse character to the sensation. The location of the pain is perceived as less superficial than that of "pricking" pain and is sometimes described as deeply penetrating, especially by some patients with lesions of peripheral nerves. A characteristic feature of this "burning" pain is its longer duration as compared with "pricking" pain.

When the duration of stimulus was less than 3 seconds, differentiation of the "burning" pain threshold from the "pricking" pain threshold was difficult. The trial exposures to the heat stimulus were well under the threshold, to demonstrate the sensation of heat without pain. This was especially important with anxious, apprehensive subjects. It was found that untrained but instructed subjects could most readily distinguish the end-point for "burning" pain with a 5- to 10-second exposure, whereas a 3-second exposure had been found most suitable for discerning a sharp end-point for "pricking" pain.

When an explanation of the technique and the sensation to be experienced was not clearly given, the subjects were doubtful what the end-point might be and were over-anxious to be cooperative and certain and therefore waited until they had experienced a "good, sharp jab of pain" before reporting an end-point. Thus, considerably more variation in the individual evaluation of the pain threshold existed without instruction as to what to observe and report, than with such instruction.

The instruction which was given to the subjects was in the same category as that given to a student to enable him to read an end-point in a titration, or in a colorimeter, or to read a Vernier Scale. In other words, it was the aim to instruct the subject as to what was the exact target he was expected to hit.

Once the instruction was presented and the observation begun, further instruction and suggestion were scrupulously avoided. The subject was not told by the operator during the tests that at this or that stage of the procedure he would experience pain, or that "now you will feel pain." In fact, no suggestion whatever was made during the period of the reading.

The method employed in these experiments for the

quantitative measurement of pain threshold was similar to that which has been used in other investigations and the procedures have been reported elsewhere (3 to 10).

I. COMPARISON OF PROPERTIES OF "BURNING" AND "PRICKING" PAIN

Observations

Series 1. The threshold for "burning" pain was ascertained on the skin of the forehead of 23

subjects. In this limited series, the variations from subject to subject were ± 16 per cent. The data for "burning" and "pricking" pain thresholds on the skin of the forehead of 11 subjects are averaged and represented in Figure 1. Invariably, on normally innervated skin, the threshold for "burning" pain was found to be lower than that for "pricking" pain.

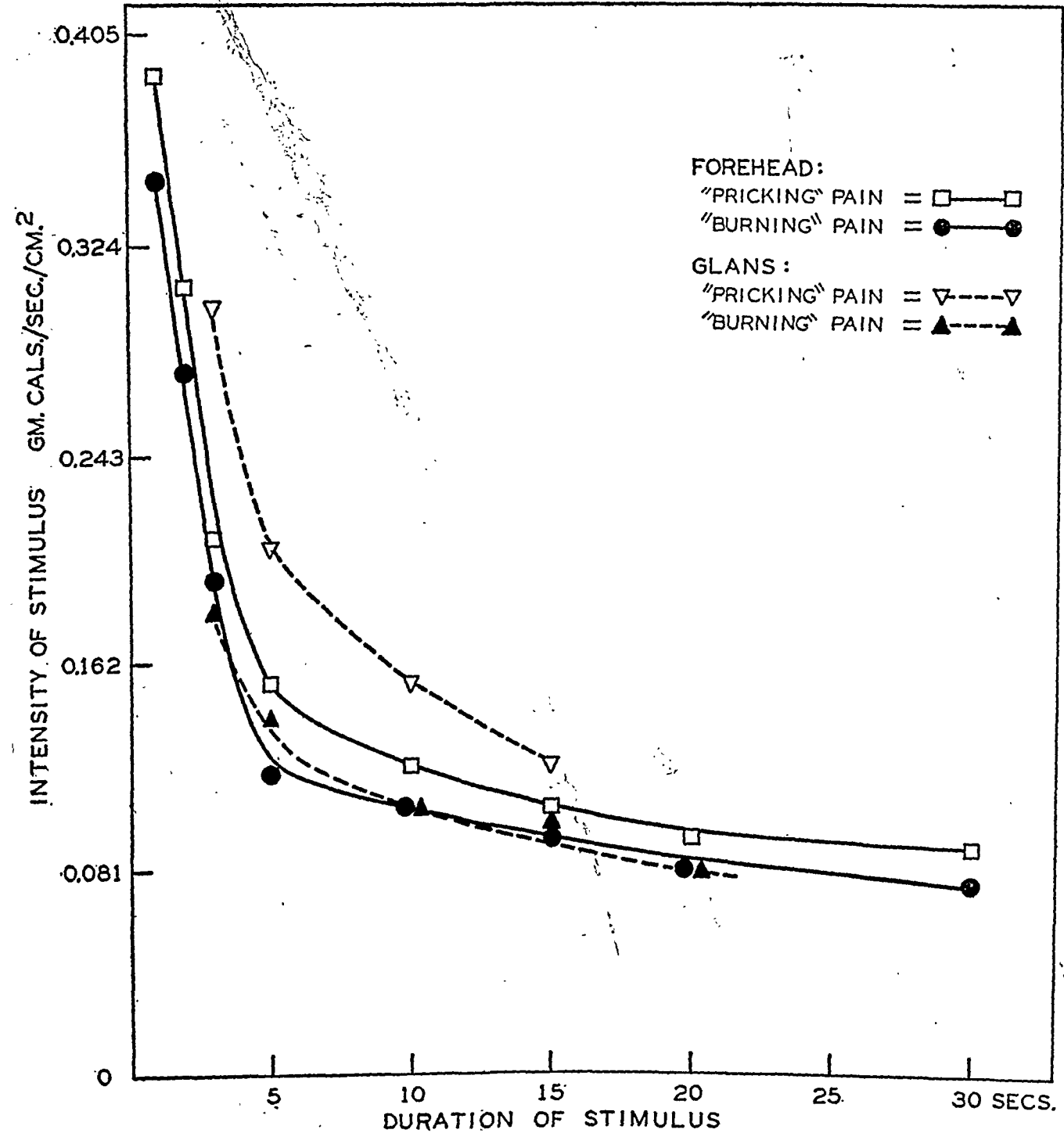


FIG. 1. THRESHOLDS OF "PRICKING" AND "BURNING" PAIN ON THE GLANS AND ON THE SKIN OF THE FOREHEAD

Series 2. The effects of analgesic agents upon the thresholds of "pricking" and "burning" pain were observed. Six observations were made on 3 subjects. It was found that ethyl alcohol (60 ml., 95 per cent) raised the threshold of both "burning" and "pricking" pain about the same percentage above their control levels. Acetylsalicylic acid also raised the threshold of both "burning" and "pricking" pain approximately the same percentage above control levels, although, to be sure, these 2 agents differed from each other in their effects (6, 9, 10).

Series 3. To ascertain whether spatial summation occurs with "burning" pain, pain thresholds were measured on areas of skin 0.4 cm.² to 11.6 cm.² in area, on 4 subjects in 5 trials. Thresholds for "burning" pain in these subjects varied no more than 25 per cent from the smallest to the largest areas of skin stimulated, which indicates that no spatial summation of significance occurs. It has already been demonstrated that significant spatial summation does not occur for "pricking" pain (3).

Series 4. Comparative observations of pain thresholds were made on the skin of the forehead and on the surface of the glans penis in 7 of the 11 normal subjects, and the data of the observations are represented in Figure 1. The "burning" pain threshold on the glans was found to be the same as on the skin of the forehead. As the intensity of the stimulation on the surface of the glans was increased on further trials, the "burning" sensation became more intense until either a "pricking" component was experienced, or the subject stated that he was unable to distinguish the sensations. With stimuli of high intensity and short duration, a distinction between the 2 end-points was extremely difficult and data about these 2 end-points under such circumstances are unreliable. From Figure 1, it is evident that the thresholds for "burning" pain both on the glans and on the skin of the forehead were approximately the same for stimuli of longer duration. The threshold for "pricking" pain on the glans appeared to be higher than on the skin of the forehead, but owing to the difficulty of perceiving "pricking" pain on the glans, these quantitative aspects of the data cannot be accepted too literally.

Series 5. Thresholds for "burning" and "pricking" pain were ascertained in 4 trials on 3

subjects who had "sunburns" or ultraviolet burn, of various degrees of severity. The threshold for "pricking" pain was reduced 43 to 52 per cent; that for "burning" pain 63 to 97 per cent from their respective control levels, ascertained on the same areas of skin on a day previous to the tissue damage (Table I).

TABLE I
Skin inflammation and pain thresholds

Subject	Area of skin injured	How Anjured	Pain threshold change	
			"Prick- ing"	"Burn- ing"
			per cent	
N. B.	Over deltoid muscle	Sunlight	-43	-63
N. B.	Over forearm	Ultraviolet lamp	-52	-97
I. H.	Over deltoid muscle	Sunlight	-51	-86
O. B.	Over deltoid muscle	Sunlight	-47	-92

Comment

Schumacher (11) has also reported the reduction of the "pricking" pain threshold of inflamed skin. The greater reduction in the "burning" pain threshold in inflamed areas of skin explains why slight stimulation of a "sunburned" skin characteristically elicits a "burning" pain sensation.

Series 6. In a patient with radiculitis, the thresholds for "pricking" and "burning" pain over an area of "hyperalgesic" skin of the left arm, were compared with normally innervated skin areas on the arms and on the forehead. The threshold for "burning" pain was about 20 per cent lower on the abnormally innervated skin area, whereas the threshold for "pricking" pain was elevated about 20 per cent (Figure 2).

A patient had "hypoalgesia" to pin prick on parts of his left hand. Rubbing the skin induced a burning sensation. At surgical operation, a displaced intervertebral disc of the sixth cervical vertebra was found. In Table II are shown the threshold measurements for "burning" and "pricking" pain ascertained on control areas of the intact hand and on the forehead, compared with thresholds ascertained on the affected hand. The quantity of radiant energy required to attain the threshold of "burning" and "pricking" pain at various time exposures is expressed in gram cal.

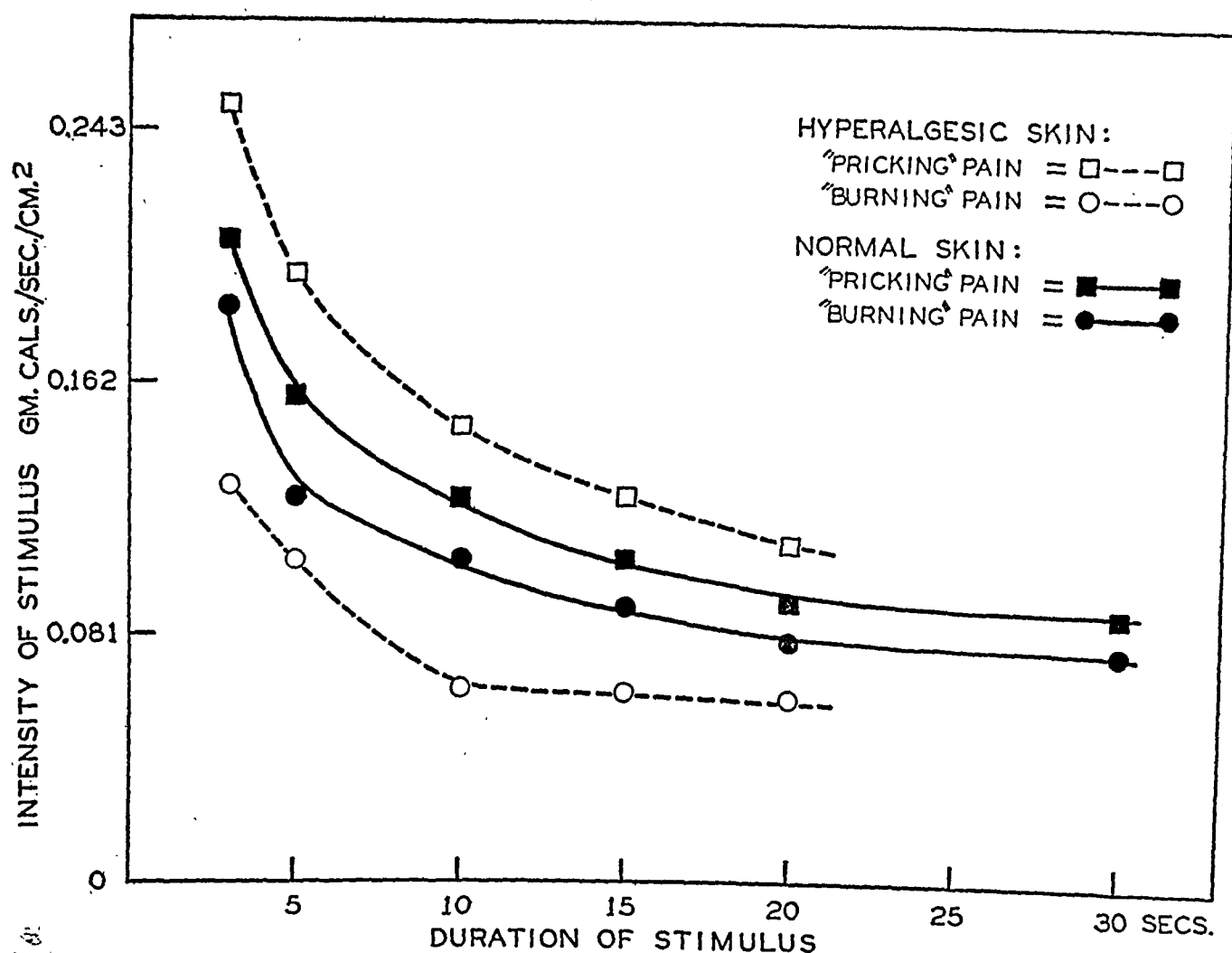


FIG. 2. COMPARISON OF THRESHOLDS OF "BURNING" PAIN TO "PRICKING" PAIN IN A PATIENT WITH HYPERALGESIA AS A RESULT OF RADICULITIS

per sec. per cm.². From this table, it can be seen that the threshold for the "burning" pain of the abnormally innervated skin was less than on control areas, whereas the threshold for "pricking" pain was higher on the abnormal skin than on control areas.

TABLE II

Thresholds for "burning" and "pricking" pain on an area of "hypoalgesic" skin compared with thresholds on control areas

Length of stimulus	Thresholds for "burning" pain			Thresholds for "pricking" pain		
	Control	"Hypoalgesic"	Deviation	Control	"Hypoalgesic"	Deviation
seconds	gram cal. per sec. per cm. ²		per cent	gram cal. per sec. per cm. ²		per cent
5	0.134	0.089	-33	0.181	0.219	+21
10	0.112	0.068	-40	0.152	0.178	+17
15	0.096	0.064	-34	0.113	0.142	+26

Series 7. Seventeen patients with "hyperalgesic" skin due to peripheral nerve disorder were observed. Three patients with herpes zoster and 14 patients with peripheral neuritis, from such varied causes as nutritional deficiency, dorsal root inflammation, and diabetes, exhibited a lowered threshold of "burning" pain, 15 to 35 per cent below control thresholds of normal skin areas on the same subject. Frequently, the "pricking" pain threshold in the affected areas was elevated.

Series 8. "Hyperalgesia" was experimentally produced in 6 subjects by occlusion of the circulation of the upper arm with a blood pressure cuff inflated to 200 mm. Hg for 35 to 75 minutes. Care was taken that the arm was not moved and that pain did not occur spontaneously. If pain developed, the experiment was discontinued. It

is known (3) that when the surface of the skin is cold, more radiant energy is required to elicit pain than when the skin surface is warm. Since the skin temperature of an ischemic arm falls gradually to room temperature, the latter was kept at 30 to 31° C. during experiments. Measurements of pain thresholds were made on the dorsum of the wrist at intervals of 2 to 3 minutes. There was observed a slight fall in "pricking" pain threshold during the first 10 minutes, followed by a rise (see Figure 3). The threshold for "burning" pain, however, fell for about 20 minutes and began to rise after 25 minutes of ischemia. Thus, between 10 and 25 minutes, the

"burning" pain threshold was depressed 28 to 32 per cent, while the "pricking" pain threshold was rapidly elevated. It was also noted that as the threshold of "burning" pain became depressed during the ischemia, the pain elicited by noxious stimulation of the skin persisted often for 5 seconds or longer, after the stimulus ended.

The persistence of pain after a noxious stimulus was most evident when the threshold of "burning" pain was most depressed. However, the "after pain" gradually diminished in duration as the threshold of "burning" pain became elevated during prolonged ischemia, and finally was not to be noted as the arm became almost insensitive.

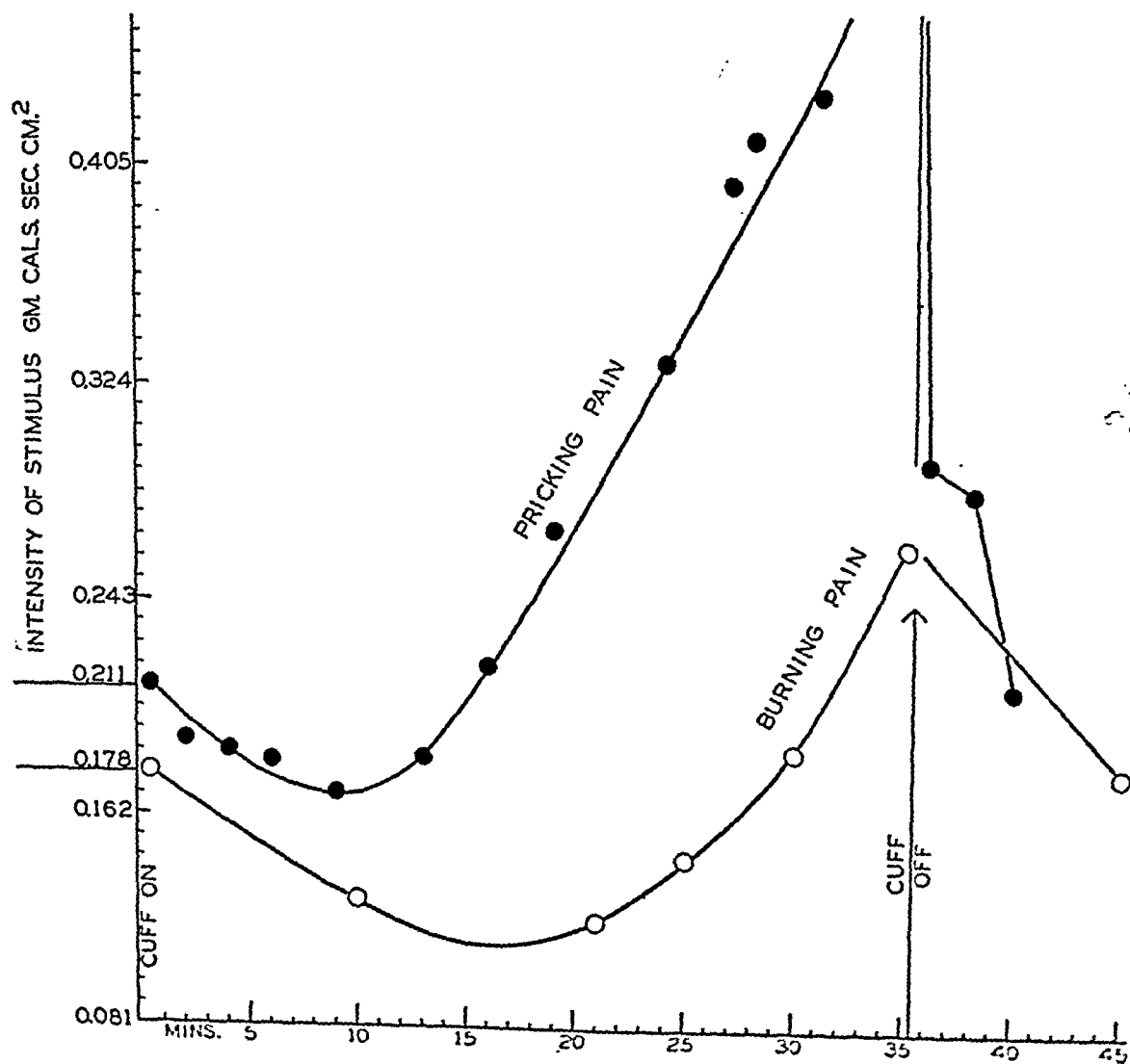


FIG. 3. ISCHEMIA AND THE PAIN THRESHOLD

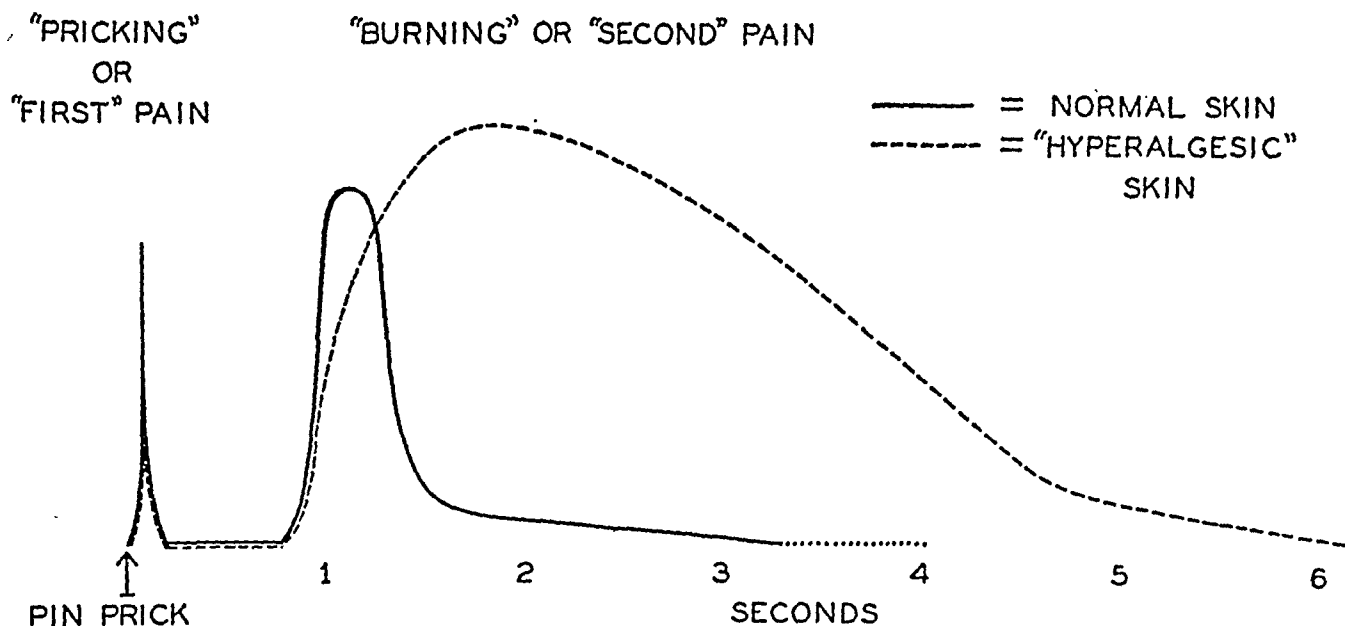


FIG. 4. SCHEMATIC REPRESENTATION OF TEMPORAL COMPONENTS OF "BURNING" AND "PRICKING" SKIN PAIN IN HEALTHY PERSONS AS CONTRASTED WITH PATIENTS HAVING "HYPERALGESIA" ASSOCIATED WITH PERIPHERAL NEUROPATHY

In one trial, when ischemia was maintained for 75 minutes, complete analgesia ensued.

II. THE DOUBLE RESPONSE

Many investigators have demonstrated that when pain is induced by pin prick or heat in the distal parts of an extremity, the sensation of "pricking" pain is followed after a painless interval by a second painful sensation. This has been called the "double response" (1, 2, 12, 13) (Figure 4). An attempt has been made in the following experiments to ascertain whether the quality of the first pain response, or rapidly perceived pain which will be designated as "fast" pain, was identical with that of the second response or the pain perceived later. The latter will be designated as "slow" pain.

Method

For purposes of pain stimulation, a sharp needle was thrust briefly and superficially into the skin surface; a brass cylinder heated to about 65° C. was briefly touched against the skin; also, the Hardy, Wolff, Goodell pain threshold apparatus was used to stimulate pain by radiant heat. These noxious stimuli, both pin prick and thermal, were applied on the distal portions of the upper and lower extremities. In a second series of observations, experimental "hyperalgesia" in normal subjects was achieved by wrapping a blood pressure cuff around the upper arm and keeping it inflated to 200 mm. Hg throughout the experiment (35 to 75 minutes). For "procaine block" a solu-

tion of procaine hydrochloride, 1 per cent, was infiltrated either into the tissues around the ulnar nerve, or subcutaneously.

Series 1. Observations on the two qualities of pain of the double response

a. Nineteen subjects noted the double response and each subject was asked whether he considered the two sensations of similar quality. Eight subjects considered that they were the same; 2 would make no decision and 9 were convinced of a difference.

b. A patient with "hyperalgesia" on his left hand, resulting from dorsal root disease, easily distinguished a double response to pin prick. He reported that the first sensation, or "fast" pain was that of a sharp pin prick, and the second, or "slow" pain was of longer duration and not "pricking." He was of the opinion that the "slow" pain was different in quality from the "fast." On testing the other, or intact, upper extremity, the double response was also noted, but here the patient was uncertain whether the two pains were different in quality.

c. A patient with "hyperalgesia," paresthesias, and wasting of the extremities resulting from peripheral neuropathy associated with nutritional deficiency, was certain that the second sensation was of a different quality from the first, describing the first as brief "pricking" and the sec-

ond as a prolonged "burning" sensation (Figure 4).

d. A patient with "hyperalgesia" of the right arm associated with a herpetiform eruption, when pricked on the affected side, described a sharp, brief "prick," followed by a prolonged "burning" sensation; on his intact arm, the two sensations were considered to be the same (Figure 4).

Comment

The reports of 19 normal subjects demonstrated again that the qualities of the two pain sensations of the double response on normal skin are not readily defined. The duration of each sensation was short and a contrast of their qualities difficult.

Boring (2) spent 15 months testing sensation on his arm and came to the conclusion that the qualities of the first and second responses differ. Lewis (13, 1), performing similar experiments with his co-workers, came to the conclusion that they were the same. The observations cited in *b*, *c*, and *d*, above, are more significant than those on normal subjects because the prolonged duration of the second or "burning" quality on patients with peripheral neuritis and dorsal root disease afforded a better opportunity for discrimination. These observations suggested again that the sensations associated with the first and second response are not identical.

In the following experiments, similar to those performed by Lewis and Pochin (12), an attempt has been made to ascertain which nerve fibers subserve "burning" and "pricking" pain sensations.

Series 2

a. In 3 subjects, ischemic block of the forearm lasting 35 to 75 minutes was produced. The "fast" pain was abolished long before the "slow" pain. A pin was used for stimulating. After 35 minutes of ischemia, no sensation was felt immediately upon impact of the pin. After about a second, however, a "burning" sensation was perceived. Pain of this "burning" quality was also produced by other means of noxious stimulation, including pinching, hair pulling, and burning. The delayed pain was always perceived as "burning."

b. In 2 experiments on one subject, a solution of procaine hydrochloride was infiltrated about the ulnar nerve. In 9 experiments on 6 subjects, procaine was infiltrated subcutaneously beneath an area of skin (5×7 cm.) on the dorsum of the hand. Observations were made during the period of incomplete analgesia: (1) immediately after infiltration and (2) as the effect of the procaine diminished.

During such incomplete analgesia, it was noted that "slow" pain was abolished while "fast" pain was retained. The quality of pin prick was not the same as that obtained on normal skin. It seemed to be sharper and less "full," as though a very fine needle were penetrating the skin. When thermal radiation was used as a noxious stimulus, the sensation was that of many tiny sharp needles being applied, similar to the familiar "pins and needles" sensation. There was no detectable sensation of "burning." The sensation perceived was of a distinctly different quality from that felt on stimulation during ischemia.

c. In 12 experiments on 5 subjects, procaine hydrochloride was infiltrated in skin areas (5×7 cm.) on the dorsum of the hand. The threshold for "pricking" pain was elevated in this area. Again, observations were made during the phase of incomplete analgesia immediately after injection, and as the action of the procaine diminished. In none of these experiments could a "burning" pain threshold be obtained during the period when the "pricking" pain threshold was elevated.

d. On 3 separate occasions in one subject, Dr. Bronson Ray infiltrated the tissue about the ulnar nerve with a solution of procaine hydrochloride, 1 per cent. The pain threshold of the skin area on the medial aspect of the wrist innervated by the ulnar nerve was ascertained immediately after the injection and during the phase of diminishing action of the procaine. In all 3 observations, the threshold for "pricking" pain was elevated,—in 1 instance, 189 per cent higher than the threshold on the control area. In this experiment, the first sensation perceived was a fine needle-like "pricking"; no "burning" pain was noted. The intensity of thermal radiation was great enough to produce a third degree burn. When the action of the procaine was ended, the subject experienced "burning" pain which persisted for 24 hours.

Comment

The observations of Lewis and his co-workers, (1 and 12) and of Gasser and his co-workers (14 to 17) have given the double response special significance. Lewis found that the "fast" pain was more readily altered by ischemia than was "slow" pain. Gasser found that the function of myelinated fibers was more readily impaired during the ischemic state than was that of unmyelinated fibers, suggesting that "fast" pain is mediated by the former. Conversely, cocaine abolishes first the action of the slower-conducting, unmyelinated fibers. Lewis noted that when either the skin or the cutaneous nerve supplying it is cocainized, the "slow" pain response is abolished, while the "fast" pain response is retained. This suggested that the "slow" pain is subserved by unmyelinated fibers. The interval between the first and second responses, which increases with the distance of the stimulus from the cord, approximates the calculated time interval based on the conduction rate of impulses traveling in myelinated and unmyelinated fibers.

It was demonstrated by the observations of this communication that ischemic neuropathy interferes more with perception of "pricking" pain than with the perception of "burning" pain. Also, during the phases of incomplete analgesia following procaine infiltration, perception of "burning" pain was abolished, whereas "pricking" pain was still perceived.

Lewis (1 and 13) is of the opinion that regardless of whether "fast" or "slow" there is only "one quality" of skin pain, and that brief noxious stimulation of the skin produces a sensation described as "pricking" whereas prolonged noxious stimulation produces a sensation described as "burning." In contrast to this thesis, therefore, it can be inferred that the "pricking" quality of pain from the skin is "fast" pain, primarily conveyed by one set of fibers, *i.e.*, myelinated fibers, whereas the "burning" quality is "slow" pain and is conveyed primarily by another set of fibers, *i.e.*, unmyelinated fibers.

III. THE QUALITIES OF PAIN IN RELATION TO DEPTH OF STIMULATION IN THE SKIN

In an investigation of the double response (18), the depth of penetration of a needle into the skin was measured and related to the qualities of pain

sensation perceived. It was found that the "fast" pain of the double response was obtained at a depth of 0.25 to 0.50 mm., while the "slow" pain was obtained at a depth of 0.50 to 1.0 mm.

Observations

Series 1. Scraping the most superficial portions of the skin of the dorsum of the hand with a fine needle gave rise to a sharp, pricking sensation. In 5 observations on 3 subjects, it was reported that when ischemia was induced in the arm for a sufficient length of time to abolish the "fast" pain or "pricking" component of the double response, scraping the superficial skin was painless. A deeper penetration of the skin with a needle gave rise to "burning" pain.

Series 2. In 7 instances in 3 subjects, perineural infiltration of the ulnar nerve with procaine hydrochloride, 1 per cent, and infiltration of a skin area (5×7 cm.) on the dorsum of the hand, were performed. It was noted that during incomplete analgesia, immediately after injection and during the phase of diminishing action, scraping of the superficial skin with a needle gave rise to a tingling, pricking sensation, while deeper penetration of the needle was at the same time painless.

Comment

Two nerve fiber plexuses in the skin have been described (19): an intraepidermal, or superficial, plexus supplied by myelinated fibers, and a subepithelial, or deeper, plexus supplied by unmyelinated fibers. Unfortunately, no conclusions can be drawn from these data concerning sensation.

Since ischemia eliminated painful responses from the most superficial portions of the skin before affecting the deeper, and since, at this time, the "fast" or "pricking" pain of the double response was also eliminated, it may be inferred that the apparatus for the "slow" pain alone was functioning, and that this lies deeper than the apparatus subserving "fast" pain.

This inference was supported by the observations made immediately following procaine infiltration of the skin, when the superficial "pricking" pain alone was perceived.

IV. INFERENCE CONCERNING THE "HYPERALGESIA" OF PERIPHERAL NEUROPATHY

The sensory changes that occur during ischemia of an extremity resemble in many respects those

which occur in the peripheral neuropathy of alcoholism with nutritional deficiencies (24), as well as in other varieties of peripheral neuropathies. Sensations subserved by the myelinated fibers are impaired before those subserved by the unmyelinated fibers.

It has been shown (Figure 3) that the ischemia was associated with first a lowering of the "pricking" and "burning" pain thresholds, and later with an elevation of both thresholds. Perhaps because it is transitory and of lesser magnitude, the phase of lowered "pricking" pain threshold in patients with peripheral neuropathy is seldom described. On the other hand, because of its persistence and greater magnitude, the lowering of the "burning" pain threshold is a striking bedside phenomenon. Perhaps relevant to these observations is the initial lowering of the threshold of stimulation of nerve fibers during ischemia and anoxia which has been noted by several investigators (20 to 22).

During the early stages of anoxia, the threshold of stimulation of the "C" group of fibers is markedly depressed, and possibly more so than is that of the "A" group. Moreover, the "C" group of fibers maintains its phase of increased irritability longer than does the "A" group (23).

During ischemic neuropathy, "burning" pain long outlasted the period of stimulation; whereas on normal skin, "burning" pain ceased soon after the noxious stimulus ended. Such persistent pain was also reported by some patients with "hyperalgesic" skin areas due to peripheral nerve disorders. In these, there was no defect in circulation; hence, it is unlikely that the persistence of pain is due to a faulty blood flow.

It is seen in Figure 3 that, after about 15 minutes of ischemia, the "pricking" pain threshold had been elevated, while the threshold for "burning" pain was still decreasing. At this time, a pin point when pressed into the skin was less "pricking" and more "burning."

One author (24) suggested that such "paradoxical pain" resulted because the loss of one type of pain sensation enhanced the perception of another and supported this view by citing another (25), i.e., "that the function of the impulses which run on ahead of the others is to adjust the excitability of the synapses in preparation for the arrival of the later impulses." Thus, according

to his views, if the impulses from myelinated fibers are lacking, there would be no synaptic adjustment and the undamped neural impulses would give rise to more intense sensation. Gasser (14 and 25), however, in the earlier formulation (14, 25) upon which the more recent worker based his views, concerned himself not with sensation, but with a simple spinal reflex pattern. He did not infer that a rapidly traveling impulse, as a result of noxious stimulation, alters a synaptic junction for another impulse from noxious stimulation so that the latter gives rise to a sensation of a different intensity or quality.

The data from the experiments of this communication suggest a simpler conception of the "paradoxical pain" of peripheral neuropathy: as the expression of a defect in peripheral nerve, the threshold for "burning" pain subserved by unmyelinated fibers is so depressed that ordinarily innocuous stimuli are perceived as painful, whereas at the same time the threshold for "pricking" pain, subserved by myelinated fibers, is elevated. Under these circumstances, contact with the bed clothes produces a painful burning, and yet pin prick perception is impaired.

V. SUMMARY AND CONCLUSIONS

1. The nature and significance of two pain sensations of the skin—"pricking" pain and "burning" pain—have been investigated.
2. The threshold for "burning" pain is lower than the threshold for "pricking" pain.
3. The peripheral nerve endings which subserve "pricking" pain are located more superficially in the skin than those subserving "burning" pain.
4. During the phase of incomplete analgesia following infiltration of procaine hydrochloride into the skin or about a peripheral nerve, "pricking" pain is perceived, whereas "burning" pain is abolished.
5. "Pricking" pain is the first or "fast" pain perceived in the double response to pin prick; "burning" pain is the second or "slow" pain.
6. Ischemic block of an extremity initially lowers the thresholds of "pricking" and "burning" pain. The threshold for "burning" pain is more depressed, and for a longer time, than the threshold for "pricking" pain. Also, during the period

of depressed "burning" pain threshold, the sensation of "burning" pain long outlasts the noxious stimulus. Prolonged ischemic block produced an elevation of both "pricking" and "burning" pain thresholds, and ultimately complete analgesia.

7. The "hyperalgesia" associated with peripheral neuropathy in patients, may be explained by the significant depression of the "burning" pain threshold. This is usually accompanied by an elevated threshold for "pricking" pain.

8. These experiments together with the work of others, make it seem likely that "burning" pain is conveyed chiefly by unmyelinated fibers, and "pricking" pain chiefly by myelinated fibers.

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THE CIRCULATION IN EXPERIMENTAL NEUROGENIC HYPERTENSION¹

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Experimental neurogenic hypertension has been successfully produced in the dog by resection or bilateral denervation of the carotid sinus with section of the aortic depressor nerves (1). There is at present general agreement that this type of hypertension persists for months and even years, though the levels of arterial pressure fluctuate widely in any given animal. In this respect, it differs from experimental renal hypertension which is characterized by a more constant elevation of the blood pressure (2); it resembles, however, the pre-hypertensive phase of essential hypertension during which the blood pressure is labile and hypertensive levels alternate with normal ones (3). Few reports have appeared in the literature on the circulation of unanesthetized animals with chronic neurogenic hypertension. The present paper is therefore concerned with the study of systemic hemodynamic alterations, including cardiac output, blood pressure, and the total peripheral resistance, and with an investigation of the renal and peripheral circulatory changes occurring in experimental neurogenic hypertension.

It has been found that, following denervation of the carotid sinus in the dog, the heart rate increased from an average of 100 beats to 240 beats per minute (4). Few observations have been published on the cardiac output of chronic hypertensive dogs. Using the Fick principle, one worker obtained an increase in the minute volume (5). Applying the same methods, others observed a rapid rise in the minute volume which was revealed by a decrease in the CO₂ difference between arterial and mixed venous blood (6). Using the cardiometer, however, the same investigators noticed a smaller heart volume following the clamping of the carotid arteries (6).

Data on the splanchnic and peripheral circulation of animals with chronic neurogenic hypertension are not available. In anesthetized dogs with

acutely denervated carotid sinuses, an increase was found in the arterial inflow to the hind limb (6) accompanied by vasoconstriction in the kidney, the spleen, and the intestine (7). No pressure changes occurred in the vena cava during the acute hypertensive phase (7).

METHODS

All experiments were performed over a period of from 5 to 11 months on a total of 6 unanesthetized female dogs. The animals were kept on a daily diet of 500 grams of Purina dog chow, fortified by 5000 units of Vitamin A, 200 units of Vitamin D, and 10 grams of Brewer's yeast. The dogs were made hypertensive according to the method previously described (8). The cardiac output was determined by the Fick principle which involves the measurement of the oxygen content of arterial and mixed venous blood as well as of the total oxygen consumption. The latter was obtained by connecting the muzzle of the animal to a waterless Sanborn metabolism machine with the help of a rubber mask (9). Four minutes following the onset of oxygen inhalation, the mixed venous blood was collected from the right ventricle by means of a catheter threaded through a 9-gauge needle which was inserted into the external jugular vein. The use of the needle made extensive exposure of the vein unnecessary. It also permitted repeated determinations within a short period of time. In every instance, an effort was made to insert the tip of the catheter into the ventricle rather than the auricle, as auricular blood of the dog is not fully mixed (10). The correct position of the catheter was determined by fluoroscopic examination. Clot formation in the catheter was prevented by a saline drip set to flow at a rate of 2.5 ml. per minute. The withdrawal of mixed venous blood was preceded by the removal of 7 ml. of a blood-saline mixture in order to avoid dilution of the sample; 10 ml. of mixed venous blood were then collected under oil. One minute later, an equal amount of arterial blood was removed under oil from the femoral artery. The blood oxygen was determined by the manometric method (11). For the measurement of right auricular pressure, the catheter was slightly withdrawn until its tip was within the auricular cavity. The zero point for the venous pressure was found by fluoroscopic determination of the position of the catheter tip. The coefficient of oxygen utilization was calculated by the formula:

$$\frac{\text{Difference in arterial-venous } O_2 \text{ content}}{\text{Arterial } O_2 \text{ content}} \times 100.$$

The effective renal plasma flow was determined by

¹ This work was supported by a grant from the Commonwealth Fund.

means of the clearance of p-aminohippuric acid (12). The renal blood flow was derived by use of the formula:

$$\frac{C_{pAH}}{100 - \text{hematocrit percentage}} \times 100^{\dagger}$$

The creatinine clearance was used to measure glomerular filtration (13). The filtrate fraction which expresses the percentage of water filtered from the plasma flowing through the kidneys, was calculated according to the formula:

$$\frac{\text{Filtration rate}}{\text{Effective renal plasma flow}} \times 100.$$

As a rule, 3 urine periods, lasting 10 to 15 minutes each, were found sufficient for the determination of the renal clearances. The renal fraction which expresses the percentage of the cardiac output perfusing the kidneys in a minute's time was obtained with the formula:

$$\frac{\text{Renal blood flow in liters per min.}}{\text{Cardiac output in liters per min.}} \times 100.$$

The blood flow through the fore-limb was measured with a metal plethysmograph, insulated with asbestos. In principle, the method of Lewis and Grant was followed (14). Volume changes were recorded on a smoked drum with a water manometer. This consisted of capillary tubing forming a writing point at one end and fused at the lower end to a hollow glass bulb which was made to float inside a piece of glass tubing of only slightly larger diameter (1.0 cm.), rising and falling with any change in the water level. This apparatus had the advantage over other volume recorders because of its ease of manufacture and its greater durability. The plethysmograph was heated by resistance coils surrounding the metal frame. All determinations were made at a constant temperature of 38° C. For the occlusion of the venous flow, a small blood pressure cuff, 3 inches wide, was inflated to a pressure of from 40 to 50 mm. Hg. At the end of each determination, the inflow curves were standardized by repeated injections of 2 ml. of water into one of the rubber tubes connected to the plethysmograph. Arterial blood pressures were recorded with the Hamilton manometer (15), which permitted accurate measurements of the systolic and diastolic blood pressures and of the heart rate. Mean pressures were calculated by planimetric integration of the area under the pressure curve.

Simultaneous determinations of the cardiac output and the mean blood pressure permitted calculations of the total peripheral resistance according to the formula:

$$\frac{\text{Mean pressure} \times 1332}{\text{Cardiac output per second}} \quad (16).$$

This formula expresses the loss of pressure head in the circulatory system in absolute units, one unit representing 1 dyne cm.² per second. As the total resistance measured the resistance to flow through the entire vascular tree, its variations from the normal control did not permit localization of occurring changes. Consequently, the resistance to blood flow in the renal vessels, and in those of the limb, were calculated separately by means of the

formula:

$$\frac{\text{Mean pressure} \times 1332}{\text{Renal blood flow or limb blood flow per second}}$$

Prior to the sectioning of the depressor fibers, a series of 2 to 5 control experiments was performed on each animal until constant values were obtained. During this control period, all determinations were carried out without interruption in 3 to 4 hours. This procedure permitted close correlation of the cardiac output and mean blood pressure with the circulation in the kidney and the fore-limb.

After the buffer nerves were sectioned, however, the blood pressure of some of the animals remained constant at significantly high levels for only 20 to 40 minutes before falling as much as 40 mm. Hg. Consequently, long tests could not be carried out during the hypertensive phase. Despite this difficulty, adequate results were obtained by determining the blood pressure along with either the cardiac output, the renal clearances, or the blood flow through the limb, inasmuch as each of these values could be determined during the period of constant elevation of the blood pressure. During the period of observation, the dogs were lying quietly on the table. As all the animals were well trained, local anesthesia was used only prior to the introduction of the needle into the jugular vein.

EXPERIMENTAL

Experiments on the cardiac output and the total peripheral resistance

Changes in the cardiac output were followed in a series of 6 animals, and are summarized in Tables I and II. In every instance, the minute volume rose 30 to 50 per cent following section of the depressor fibers. This change was associated with the high oxygen content of the mixed venous blood, resulting in a decrease of the arteriovenous oxygen difference. Similar observations have been made in animals with chronic neurogenic hypertension (5), and in acute experiments (6). In 4 animals, the oxygen consumption increased an average of 30 ml. of oxygen per minute. As previously observed by a number of investigators, the heart rate rose an average of 85 beats per minute in the hypertensive dogs (4). As a result of this tachycardia, the cardiac output increased, while the systolic discharge did not change from its control values. The utilization of oxygen declined in every instance following the establishment of neurogenic hypertension (Figure 1). The decrease in both arteriovenous oxygen difference and oxygen utilization was the result of the increased circulatory rate. As seen in Tables I and II, the total peripheral resistance rose sig-

TABLE II

Representing the arithmetic means of data obtained before and after sectioning of the moderator nerves

Before operation							After operation							
Dog	Mean blood pressure	Minute volume	Pulse rate	O ₂ A-V difference	Limb blood flow	Resistance		Mean blood pressure	Minute volume	Pulse rate	O ₂ A-V difference	Limb blood flow	Resistance	
						Renal	Periph-eral						Renal	Periph-eral
	mm. Hg	ml. per min.		ml. per liter blood	ml. per 100 ml. per min.	dynes cm. ⁻⁵ sec.		mm. Hg	ml. per min.		ml. per liter blood	ml. per 100 ml. per min.	dynes cm. ⁻⁵ sec.	
3	129	3513	122	61	3.9	15,000	3133	184	4913	172	41	11.8	27,000	3190
5	105	3133	122	56	6.6	32,300	2710	182	4360	169	46	15.6	48,300	3666
7	127	3340	141	63	5.0	24,800	2663	184	4603	185	55	13.5	45,500	3433
16	134	3578	105	52	4.1	21,900	3078	188	5300	183	37		39,300	3020
17	153	4750	110	44	5.9	26,800	2560	189	7050	191	36	7.5	31,800	2210

nificantly in only 2 animals (No. 7 and No. 5), but remained constant in the rest of the dogs (Nos. 1, 2, 3, 17). It has been mentioned above that the total peripheral resistance measured the resistance to flow throughout the entire organism. Therefore, its values furnished no information concerning the localization of vascular changes which took place following the section of the buffer nerves.

In every instance, the measurement of the cardiac output was followed by a determination of the auricular pressure. As seen in Table I, no change in the intra-auricular pressure occurred subsequent to the development of hypertension. In experiments on anesthetized animals, it was found that the pressure in the inferior vena cava remained at its control level (7). This observation suggested that the static volume in the veins during hypertension was the same as that existing during the control period.

Experiments on the renal circulation and the renal resistance

The rôle of the kidney in experimental neurogenic hypertension has been the subject of a series of investigations. Certain workers (17, 18) have noticed that, in sympathectomized animals with sectioned depressor fibers, arterial blood pressure decreased temporarily following renal denervation. Others (19), however, made the observation that bilateral nephrectomy failed to prevent the development of acute neurogenic hypertension. A series of 36 experiments was therefore performed in which the glomerular filtration rate and the renal

blood flow were examined both before and after section of the depressor fibers. Following the operation, both the renal blood flow and the glomerular filtration rate fell in 2 animals, but remained constant in the rest of the dogs. Because the creatinine and p-aminohippuric acid clearances fell proportionately in these 2 instances, the filtrate fraction remained at its control level, indicating that there was constriction of the afferent arterioles (20) (Figure 2). In human subjects, similar changes of the filtration fraction during orthostatic vasoconstriction have been observed (21). Constriction of the afferent arterioles seen in the experiments described above distinguished the renal circulation in neurogenic hypertension from that of essential hypertension, in which the filtrate fraction rises due to efferent arteriolar spasm (3). Arteriolar constriction is further illustrated in Figure 2, which shows a 50 per cent rise in the renal resistance and a significant fall in the renal fraction. Similar results were obtained in the rest of the animals (Tables I and II).

The circulation through the fore-limb

The evidence presented in this paper furnished no proof that renal vasoconstriction was accompanied by arteriolar spasm in other regions of the splanchnic circulation. It has been shown by others, however, that acute neurogenic hypertension was followed by vasoconstriction in the intestine and the spleen (7). Hence the assumption might be ventured that neurogenic hypertension resulted in generalized constriction of the vessels supplied by the splanchnic nerves. It seemed of

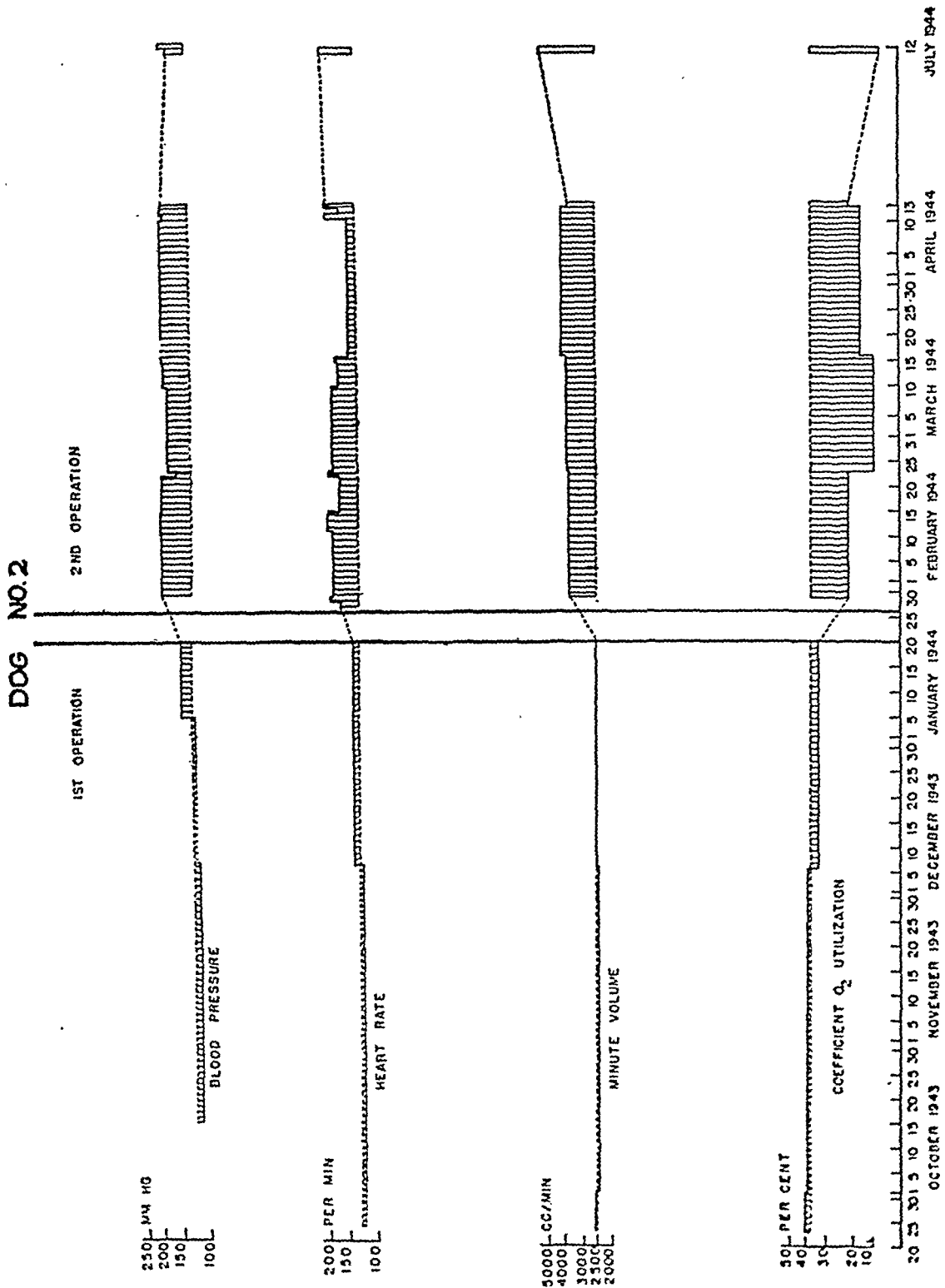


FIG. 1. ILLUSTRATES THE DEVELOPMENT OF NEUROGENIC HYPERTENSION FOLLOWED BY AN INCREASE IN THE HEART RATE AND THE MINUTE VOLUME, SIMULTANEOUSLY THE UTILIZATION OF OXYGEN DECREASES

importance, therefore, to study the blood flow through the limb in order to ascertain whether or not the increase in the minute volume reported above was to some measure accounted for by an acceleration of the blood flow through the vessels of the limbs.

Tables I and II show that the onset of hypertension was followed in 4 animals by a considerable rise in the blood flow through the forelimb, averaging 12 ml. per minute per 100 ml. of limb volume. This observation is illustrated in Figure 3, which demonstrates that the slope of the

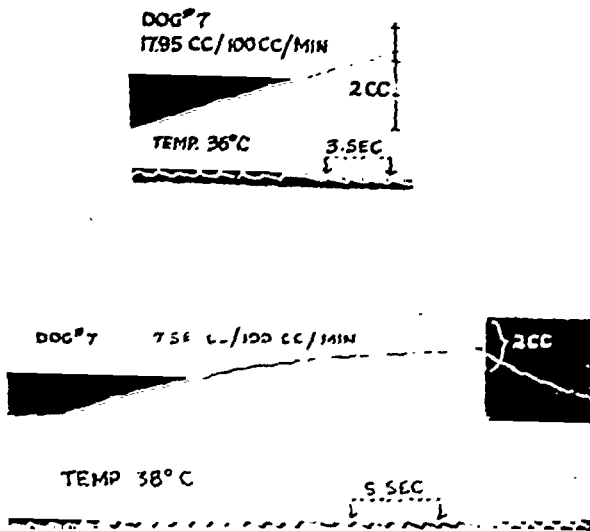


FIG. 3. SHOWS THE CURVES REPRESENTING ARTERIAL FLOW INTO THE FORE-LIMB OF A DOG

The blood flow through the fore-limb increases from 7.55 ml. to 17.95 ml. per 100 ml. of limb volume per minute after sectioning of the moderator nerves.

arterial inflow curve steepened after section of the depressor fibers. These findings are in agreement with other reports which show a significant increase in the blood flow through the femoral artery of anesthetized animals with acute carotid sinus hypertension (6).

The determinations of the vascular resistance in the limb furnished additional proof of an increase in the blood flow through the limb, the values falling an average of one million and a half absolute units (Figure 4). It was evident, therefore, that neurogenic hypertension was accompanied by an acceleration of the circulation through the extremity.

DISCUSSION

The foregoing experiments demonstrate that the production of neurogenic hypertension in unanesthetized dogs is followed by a decrease in the oxygen difference between arterial and right ventricular blood (Tables I and II), as well as, in 4 animals, by a slight rise in the oxygen consumption. Both these changes are the result of an increase in the minute volume which occurs in every instance. Similar results have been obtained by others (5, 6) in anesthetized dogs. Since the output per beat remains constant, even declining in some instances, the increase in cardiac output is dependent upon the increased heart rate. The fall in systolic discharge observed in several experiments may explain the finding of the last mentioned author that the cardiac volume decreases in some of his acutely hypertensive animals. The tachycardia which follows section of the depressor fibers permits the heart to cope with the increase in venous return and thus prevents a rise in the right auricular pressure. Increase in the circulatory rate precludes the extraction of large amounts of oxygen by the tissues and thus explains the decrease both in oxygen utilization and in arteriovenous oxygen difference. The values calculated for the total peripheral resistance vary from animal to animal, being elevated in 2 cases and showing no change in the remainder (Figure 4). This observation is of great significance, as it demonstrates that the hypertension produced by sectioning of the buffer nerves is almost entirely caused by a rise in the cardiac output.

Studies on the renal circulation of animals with neurogenic hypertension reveal a considerable degree of vasoconstriction. This is demonstrated by the observation that the renal blood flow remains constant or falls slightly, following the establishment of hypertension (Figure 2). Although renal ischemia is entirely absent or only slight, renal vasoconstriction is nevertheless present, as illustrated by a rise in renal resistance and a fall in renal fraction (Figure 2). According to the concept of Smith (20), the constriction is in the afferent arterioles, for the filtrate fraction remains unchanged. Consequently, the renal circulation in animals with neurogenic hypertension differs from that observed in essential hyperten-

sion in which a rise of the filtrate fraction indicates participation of the efferent arterioles (4). It is probable that the changes in the renal circulation described above are mediated by the sympathetic nervous system. This conclusion is supported by the observation of Smith that afferent constriction is brought into play by orthostatic changes in man which are known to be the results of sympathetic discharges (21).

The question whether vasoconstriction occurs in parts of the splanchnic circulation other than the kidney cannot be answered with certainty at the moment. If changes in the renal circulation follow those in other parts of the abdominal cavity, the assumption might be ventured that chronic neurogenic hypertension is accompanied by generalized splanchnic vasoconstriction. This conclusion is borne out by certain investigations which showed vasoconstriction in the intestine and spleen during the acute hypertensive phase (7).

The response of the vessels of the extremity to sectioning of the depressor fibers contrasts sharply with that of the renal arterioles: there is a rise in the blood flow through the fore-limb and a fall in the resistance in this extremity (Figure 4, Tables I and II). The above mentioned workers also describe an increased rate of flow through the femoral artery of anesthetized animals with acute neurogenic hypertension (6); they are of the opinion that this hyperemia is caused by the opening of arteriovenous shunts. On the basis of the results reported in this paper, it is impossible to conclude whether or not the peripheral hyperemia exists because of the opening of arteriovenous anastomoses, or by active arteriolar vasodilation.

The acceleration of the circulation through the limb furnishes additional evidence of increased sympathetic activity in neurogenic hypertension. Sympathetic vasodilation in the leg of the dog has been demonstrated successfully (22, 23). In man, other workers found that the subcutaneous injection of epinephrine was followed by marked vasoconstriction in the hand and foot, and by a definite increase in the blood flow through the forearm and calf (24). These investigators conclude that epinephrine causes vasoconstriction in the skin and active vasodilation in the muscle. It has been noticed in man that the intravenous injection of adrenalin produced a rise in muscle tem-

perature and a fall in skin temperature (25). The difference in the reaction between the vessels of the skin and those of the muscles could explain the fact that in one animal (No. 17) the blood flow through the limb rose only slightly (Table II). In this dog, the large size of the leg permitted the inclusion of only relatively muscle-free paw in the plethysmograph.

The vascular dynamics of neurogenic hypertension differ in many respects from those observed in experimental renal hypertension. In Goldblatt hypertension, and in essential hypertension, the cardiac output is normal (26, 27); and the total peripheral resistance is increased (28). Other studies (29, 30) demonstrate that in essential hypertension the peripheral blood flow is not elevated despite the rise in systemic blood pressure. The renal circulation in essential hypertension is characterized by afferent arteriolar constriction which extends also to the efferent arterioles (4), in contrast to the afferent arteriolar constriction demonstrated in the experiments reported in this paper. In experimental renal hypertension and in hypertensive human subjects, the increase in blood pressure is the result of a generalized decrease in the arteriolar cross-sectional area. In neurogenic hypertension, however, it is due to an increase in the cardiac output which exists in conjunction with a shift in blood flow to the extremities.

SUMMARY

1. The hemodynamic alterations during chronic neurogenic hypertension were studied in 6 unanesthetized dogs.

2. Following the establishment of hypertension, the cardiac output rose, while the difference in the oxygen content between arterial and mixed venous blood, and the co-efficient of oxygen utilization, decreased. Since the heart rate increased, the systolic discharge and the right auricular pressure remained at their pre-hypertensive levels. The total resistance showed no change in 4 dogs, while it rose in the remainder of the animals.

3. The blood flow through the kidney and the glomerular filtration rate fell in 2 animals and remained constant in the rest. The decline in the renal fraction and the rise in the renal vascular resistance are evidence of renal arteriolar con-

striction. This takes place presumably in the afferent arterioles as the filtrate fraction remained constant.

4. The development of neurogenic hypertension was followed by a marked rise in the blood flow through the fore-limb and a fall of the vascular resistance through this extremity.

5. The changes described in this paper are compatible with increased sympathetic tone.

6. Differences in the vascular dynamics between neurogenic hypertension, experimental renal hypertension, and essential hypertension, are discussed.

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NITROGEN METABOLISM IN ACUTE INFECTIONS¹

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Wastage of nitrogen in severe infectious diseases has long been recognized. More recently, it has been discovered that surgical operations and trauma induce the same process. Much evidence has accumulated that, in the acute stages of all these conditions, the losses of nitrogen can be prevented or mitigated with difficulty, if at all, by the administration of unusually large quantities of protein or high calories, or both. The subject has been recently reviewed by one of the authors (1).

On *a priori* grounds, losses of nitrogen, which must be derived from proteins of the tissues, would seem to be undesirable. It has been generally assumed that the body has no surplus stores of protein and, consequently, that losses sustained through disease, injury, or deprivation must be replaced before a subject is restored to full health and strength. If this is true, it becomes a matter of no small importance to find a means to prevent these losses and thus to hasten convalescence and rehabilitation.

Studies of the subject have been confined chiefly to major diseases and injuries. Obviously, however, from the standpoint of conservation of manpower, the ordinary run of the mill illnesses and injuries are of the greatest importance, because it is the victims of these that may be returned most rapidly to normal life. The present study is aimed to measure the nitrogen excretion in such common conditions and to ascertain how far nitrogen wastage may be prevented by dietetic measures. Since it is obviously unpractical to use parenteral or other abnormal methods of feeding for all patients in the hospital, such methods have been practised in this study for only one purpose: to learn whether the administration of supplementary protein to the diet of a patient would pre-

vent nitrogen losses that could not be prevented by dietary measures alone.

EXPERIMENTAL PROCEDURE

Patients on the wards of the New Haven Hospital were used, young adult males being selected, as far as possible. One female patient, JK, was included. Studies were terminated as soon as patients came into positive nitrogen balance or when they were discharged from the hospital.

Chemical procedures. Urine, feces, vomitus, and in one case sputum, were analyzed for total nitrogen by the macro-Kjeldahl technique. Twenty-four-hour urine specimens, preserved with benzoic acid, were analyzed daily. Stools, collected in Pyrex glass beanpots, were quantitatively transferred to jars containing benzoic acid. The combined stools were analyzed at convenient intervals. The quantity of nitrogen in each specimen was divided by the number of days over which it had been collected and the average thus obtained was used for calculation of the daily nitrogen balances in that period. The total fecal mass, diluted with water to a convenient weight and consistency, was thoroughly mixed with a motor-driven mechanical stirrer for at least 20 minutes. Aliquots, obtained while the stirrer was running, were transferred to weighed Kjeldahl flasks, which were then reweighed. Vomitus and sputum were analyzed separately by similar procedures.

Blood was collected before breakfast at the beginning and end of each experiment. The non-protein nitrogen of whole oxalated blood was measured by the micro-Kjeldahl technique. Total protein and protein fractions were measured in serum from blood drawn and centrifuged with anaerobic precautions. Cell volume was measured by hematocrit on blood defibrinated anaerobically with mercury. All analyses were made in duplicate and were repeated when duplicates did not check.

Diets. Liquid, soft, or regular diets were used, according to the condition of the patients. The basic liquid diet was prepared to contain 100 grams of protein and 3000 Calories. A mixture made by adding 10 grams of skimmed milk powder to 100 ml. of milk and flavoring with a few drops of vanilla was found useful. Each liter of this mixture contained 67 grams of protein and provided 900 Calories. When well mixed, it was quite palatable, tasting like an ordinary milkshake. The basic soft and regular diets were prepared to contain 125 grams of protein and 3500 Calories. Uneaten portions of each meal were weighed and the amounts deducted from the diets offered.

The nitrogen in the diets was calculated by means of

¹ The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Yale University School of Medicine.

the usual dietary charts. These calculations were, however, checked by weekly analyses of duplicate diets by the macro-Kjeldahl technique. The diet, ground up by a meat grinder was suspended in water and treated in the manner described for the analysis of stools above.

The liquid diets, instead of the calculated 16 grams of N, were found, on 6 analyses, to contain 12.6 to 15.9 grams; the solid diets, instead of 20 grams, contained, on 15 analyses, from 16.5 to 22.6 grams of N.

General care. Special nurses were in attendance upon the patients throughout. This not only insured accuracy in the administration of food and the collection of specimens; it also contributed to success in feeding. Many of the patients would have taken far less food during the more severe stages of their illnesses, had it not been for the skillful services of these nurses.

Except for the administration of food and fluids and attention to general comfort that might be conducive to appetite, the care of the patients was left to the regular physicians and surgeons of the hospital staff. Supervision of the details of the project and liaison between the laboratory and wards were entrusted to a special physician who spent his full time on the project.

Amigen, a hydrolyzate of casein and pork pancreas, prepared by Mead Johnson and Co.,¹ was given intravenously to certain of the patients, in 5 per cent solution with 5 per cent glucose. Doses of 500 ml. were injected slowly over a period of 2 to 3 hours. It was assumed that such a dose contained 6 grams of N.

Amigen was given only when patients took little or nothing by mouth, as supplements to the diets when the N-intake was obviously inadequate, or when it was known that there was a large negative nitrogen balance. In the last case, Amigen was given on single days in order that the effect of a large addition of nitrogen upon the nitrogen balance might be evaluated.

OBSERVATIONS

Balance studies were conducted on 45 patients. The duration of studies on individual patients varied as follows: 7 patients were observed for only 2 to 3 days, because they were discharged at the end of this period, with 1 exception in positive nitrogen balance; 37 patients were observed from 4 to 22 days; one was observed 43 days.

There were 22 patients with medical conditions: 10 meningococcus meningitis (2 with complicating polyarthritides), 2 pneumococcus pneumonia, 2 scarlet fever, 3 upper respiratory infections, and 1 each of subacute bacterial endocarditis, lung abscess, peritonsillar abscess, acute rheumatic fever, and regional enteritis. Acute febrile illnesses studied as they were admitted. Meningococ-

¹Amigen used for this study was supplied by Mead Johnson and Co.

cus meningitis happened to be the prevalent major infection at the time of the study.

The remaining 23 patients had surgical conditions: 9 appendectomies, 3 herniorrhaphies, 3 fractures of long bones, 2 skin infections, 2 chronic osteomyelitis, 1 saphenous vein ligation, 1 radical mastoidectomy, 1 traumatic rupture of the iliopsoas muscle with evacuation of an extraperitoneal hematoma, and 1 excision and curettage of a bone cyst.

Amigen was given to 4 of the patients with meningitis, 6 appendectomies, 2 fractures, 1 herniorrhaphy, and 1 regional enteritis. In the surgical patients, it was used when the patients could take little or nothing by mouth.

Table I summarizes the data from all cases. The first column indicates the day of the disease on which the study was begun. In the operative cases, the days are numbered from the day of operation, except in the case of LK, in which the study was begun 5 days after injury or 2 days after operation. The total duration of each study is the sum of the days in positive and in negative balance (PSe and AI were each studied one day longer than this, but in each case one day was omitted because urine collection was incomplete). In all cases except PSe, KS, and JK, the days of positive nitrogen balance were at the end of the study, following a variable period of negative balance. In these 3 patients, days of positive and negative balance were interspersed. The temperature column shows the number of days on which at least one temperature reading was 100° F. or more by rectum, or 99° or over by mouth. In operative cases, a double set of nitrogen balance figures is given for those patients who took little or nothing during the period immediately after operation. The upper figures represent the nitrogen metabolism for this period, the lower figures, the nitrogen metabolism for the remainder of the period of negative balance.

Figures 1 and 2 show the complete nitrogen metabolism of 5 of the meningitis cases and 8 of the appendectomies. The nitrogen balances of 3 other appendectomy cases are shown below Figure 2. The nitrogen intakes of these 3 patients after the first few postoperative days were adequate and fairly uniform, averaging well over 14 grams daily. The cases presented in detail in

TABLE I

Case	Initial day of disease	Nitrogen							Temperature	Signs and symps.	Change of serum		Diagnosis
		Total balance	Lost			Stored					Albu- min	Glob- ulin	
			Daily	Number of days	Average intake	Daily	Number of days	Average intake					
		grams	grams		grams per day	grams		grams per day	no. of days		grams per cent		
EB	4	-40	4.0	10	14	0	0		2	2	+0.6	+0.3	Meningitis
AI	4	-29	7.3	4	12	0	0		1	1	+0.3	-0.2	Meningitis
PS ^h	5	-10	2.5	4	16	0	0		0	0	+0.2	-0.2	Meningitis
JF	6	-66	7.4	9	16	0.9	1	19	6	5	+1.2	+0.2	Meningitis
PV	5	-123	11.2	11	14	0	0		6	7	+0.5	+0.9	Meningitis
TF	4	-31	6.2	5	18	0	0		3	2	+0.2	+0.1	Meningitis
FC	9	-38	5.5	7	16	0.9	1	29	4	4	+0.3	+0.6	Meningitis
PS ^e	4	-3	2.2	5	16	2.1	4	22	4	3	-0.3	+0.7	Meningitis
HP	3	-180	8.2	22	14	0	0		18	18	+0.5	+1.4	Meningitis with poly- arthritis
ND	3	-126	7.0	19	16	2.3	3	16	18	17	+0.3	+0.7	Meningitis with poly- arthritis
WZ	8	-25	3.3	8	15	1.6	1	18	2	2	+0.9	+1.0	Pneumonia
PS ^m	6	+9	0	0	0	2.3	4	17	1	0	+0.4	+0.5	Pneumonia
RG	2	-34	3.8	10	17	0.9	4	18	4	2	+0.4	+0.2	Scarlet fever
GN	5	-18	3.1	7	18	1.0	3	18	0	0	+0.6	+0.2	Scarlet fever
TP	56	+19	0	0		2.3	8	14	3	8			Lung abscess
FS	131	+4	0	0		1.4	3	14	3	3	-0.1	+0.2	Subacute bacterial endo- carditis
ES	17	+2	0	0		0.8	3	19	3	3			Sepsis, following wound infection
HD	43	+34	0	0		4.8	7	17	0	5	+0.8	0	Osteomyelitis
KS	43	+17	-0.7	5	18	1.7	12	14	1 ²	4			Rheumatic fever
MH	1 ¹	+7	0	0		2.2	3	17	1 ²	0			Saphenous vein ligation
GB	2 ¹	-24	-6.0	4	14	0	0		3 ²	4	+0.3	+0.1	Mastoidectomy
AM	Op. ¹	-1	7.8 -0.6	1 4	1 14	1.2	8	17	6 ²	3	0	-0.1	Herniorrhaphy
JD	1 ¹	-20	-20.1 -1.8	1 5	0 18	1.4	7	19	3 ²	1	-0.1	+0.4	Herniorrhaphy
WT	Op. ¹	+4	-3.6	3	13	1.8	8	18	3 ²	2	-0.3	+0.3	Herniorrhaphy
JK	Op. ¹	+10	-6.3 -2.4	1 5	3 14	2.0	14	17	18 ²	14	-1.1	+0.7	Cyst of femur operation
LK	5	-111	-19.1 -6.0	2 18	3 18	0	0		11 ²	5	-0.4	+1.3	Hematoma
HM	3	-93	6.2	15	18	0	0		7 ²	15	0	+1.1	Fracture of femur
JC	2	-95	11.9	8	17	0	0		3 ²	8	-0.4	+0.6	Fracture of ribs, tibiae and fibulae
JG	2	+16	0	0		5.4	3	20	2 ²	3			Fracture of tibia
ED	1	-25	12.9 2.1	1 6	1 18	0	0		1 ²	1	+0.5	+0.4	Appendectomy, acute appendicitis
RW	Op. ¹	-52	10.3 3.5	3 6	1 16	1.6	1	16	5 ²	5	0	+0.3	Appendectomy, acute appendicitis
HY	Op. ¹	-31	9.0 1.7	2 8	0 15	1.0	1	14	1 ²	2	-1.0	+1.2	Appendectomy, normal appendix
PM	1 ¹	-49	6.1	8	14	1.9 ⁴	1	17	5 ²	2			Appendectomy, normal appendix
VP	1 ¹	-23	3.8	7	14	1.0	3	18	4 ²	2	-0.4	+0.8	Appendectomy, acute appendicitis
JD	1 ¹	-13	6.0	4	14	2.3	5	19	4 ²	2	-0.4	+1.1	Appendectomy, acute appendicitis
AS	Op. ¹	-12	3.7	4	18	1.0	3	16	3 ²	2			Appendectomy, normal appendix
RS	Op. ¹	+15	2.5	2	12	2.6	8	18	2 ²	2	-0.5	+1.1	Appendectomy, acute appendicitis
TL	1 ¹	-110	4.1	31	16	1.5	12	17	42 ²	43			Appendectomy, acute appendicitis, compli- cated by pneumonitis and wound infection

¹ Days counted from day of operation.² Patient on large doses of salicylates throughout course.³ Temperature taken orally, 99° F. or over.⁴ Patient failed to complete 24 hour specimen; otherwise this day's balance would probably have been negative.

these figures were those who received injections of Amigen.

DISCUSSION

A large proportion of all patients wasted nitrogen to a variable degree. This wasting bore some relation to the type and severity of the disease; but losses varied to some extent among patients with the same disease and from day to day in individual patients. In many instances, they could not be prevented by increasing nitrogen and caloric intakes within feasible limits. They frequently persisted after patients had become asymptomatic. There was no consistent relationship between these losses and the changes of serum albumin or globulin.

Losses of nitrogen in meningitis were surprisingly large and prolonged. They varied, during the periods of study from 3 grams in PSe to 180 grams in HP, who had a complicating polyarthritis with prolonged fever. This loss, equivalent to about 4.5 kgm. of muscle tissue was accompanied by a loss of 7.5 kgm. of body weight. The other patient with polyarthritis, ND, also wasted a large amount of nitrogen. PV, however, lost almost as much, 123 grams, without serious complication. Of the whole series, only PSe stored a significant quantity before discharge from the hospital. ND retained a small amount in the last 3 days before discharge; FC and JF had equivocal retentions on the last day. The remainder were still wasting when they were well enough to leave the hospital, from 8 to 25 days after the onset of illness. The losses tended to diminish as convalescence progressed; but TF and PV lost more than 12 grams each during the 2 days prior to discharge. The diets in all but one instance contained an average of 14 grams or more of nitrogen throughout the studies; during the last days of hospitalization they were usually considerably above this. One of the patients with pneumonia, WZ, behaved in a similar manner, losing 25 grams of nitrogen from the 9th to the 17th day after the onset of his disease inclusive, although his temperature was normal after the 10th day.

More surprising are the losses in the patients with scarlet fever. These 2 subjects were afebrile and asymptomatic during the greater part of the studies; they were merely awaiting termination of their periods of isolation. From the figures for

storage it is evident that replacement of the wasted nitrogen was a slow process.

In contrast to these subjects, 4 patients with long standing infections that had produced considerable wasting, TP, FS, ES, and HD, stored nitrogen throughout their studies on diets comparable to those on which the patients with acute infections wasted nitrogen. This is similar to the phenomena which others (2, 3) have described in persons with fractures and other severe injuries. These subjects waste nitrogen for a considerable time after the original injury. Only after malnutrition has become advanced do they gradually reverse the process. If, during the reparative or anabolic stage, they suffer renewed injury or infection, the process of storage of nitrogen may continue uninterrupted (2).

Minor respiratory infections, not shown in the table or figures, did not disturb nitrogen metabolism appreciably.

Surgical procedures *per se* had no uniform effect upon nitrogen balances. There were, of course, inevitable losses on the operative and immediate postoperative days while patients received little or no nourishment. Patients who had appendectomies continued to lose nitrogen for a few days after this period in spite of more than adequate intakes and uneventful courses. This was apparently uninfluenced by the pathological state of the appendix. In spite of the fact that their appendices were normal, HY, PM, and AS lost variable amounts of nitrogen; while among the others, with different degrees of inflammation, the nitrogen balances were variable, one, RS, even storing nitrogen from the outset. GB after a mastoidectomy also wasted nitrogen, exclusive of the drainage which would have increased the negative balance had its nitrogen content been included. On the other hand, patients subjected to herniorrhaphy tended to store nitrogen as soon as they had passed the immediate preoperative period and were given adequate diets. JK, who had a bone cyst excised, and MH, after ligation of the saphenous veins, lost no nitrogen. The patient LK, with the extraperitoneal hematoma, lost large amounts of nitrogen. Of the 3 patients with fractures, 2, with fractures of the femur, and of ribs, both tibiae and fibulae respectively, followed the course described by others (3), wasting nitrogen as long as they were under observation; the third,

with a comminuted fracture of the tibia, stored small amounts of nitrogen from the beginning, although his diet was not much larger than those of the 2 subjects who wasted nitrogen.

Loss of nitrogen in the patients with acute infections was not definitely related to the febrile reaction. It is true that the largest losses in meningitis occurred in the early febrile days and continued longest in the 2 cases with polyarthritis and prolonged fever. Nitrogen continued to be wasted, however, by almost all the patients after the temperature had become normal. PV lost as much nitrogen in 5 afebrile days as he had during 6 days of fever, on several of which the temperature did not rise above 100° F. The patients with chronic infections stored nitrogen despite elevated temperatures. The apparent correlation between temperature and negative nitrogen balances probably indicates merely that both phenomena are marks of the severity of illness.

The stools did not contain excessive amounts of nitrogen. About 2 grams of N per day were found in the sputum of JC with fractures of ribs and legs.

It has been suggested that the nitrogen loss in disease arises from the atrophy of disuse, induced by bed rest. If this were the case, there is no reason why herniorrhaphies should not have the same effect as appendectomies. Furthermore, nitrogen losses should not have persisted so far into convalescence in either meningitis or scarlet fever. Finally, some of the patients observed preoperatively did not waste nitrogen when confined to bed nor did some of those with acute infections, injuries, or operations.

Increasing the nitrogen intake with food or with Amigen supplements had no consistent effect. Daily intakes of over 21 grams of food nitrogen and over 25 grams of food + Amigen nitrogen failed to establish positive balances in some instances (see, for example, TF 8th day and FC 13th day in Figure 1). On other occasions (*e.g.*, PSe in Figure 1), raising nitrogen intake sharply by means of Amigen injections seemed to mitigate or prevent losses of nitrogen. The data must be interpreted with some reserve, however, for several reasons: first, urinary nitrogen in most cases varies capriciously; second, subjects with apparently similar conditions differ in their tendency to waste nitrogen; third, the duration of the

negative balance is variable; finally, there may be a delay in the excretion of nitrogen. Most of these points are illustrated in the figures. In TF, Figure 1, losses of nitrogen seem to be inversely proportional to intake, Amigen supplements apparently spare nitrogen, until the 8th day when a sudden large negative balance is associated with the largest nitrogen intake that was achieved. In most of the other cases in both figures, variability of nitrogen outputs without relation to diet is evident. After the 10th day, losses of N by JF, Figure 1, seem to diminish as the dietary N increases. This is only a mark of general improvement, evidenced in better appetite. There is a positive balance on each of the days on which PSe, Figure 1, received Amigen supplements, with a negative balance on the alternate days. Since, however, the supplements were injected after the evening meal, a proportion of the injected nitrogen may have been excreted in the urine of the following day.

The administration of 12 grams of Amigen per day to surgical patients on the day of operation and the following day, when they received no other food, seemed to prevent losses of nitrogen for 1 day in both RS and AS. The positive balance on the operative day of AS must be discounted, however, because he had difficulty in emptying his bladder. On the whole, the losses of nitrogen in these patients who received Amigen (see detailed charts of Figure 2) were not significantly smaller than those of the patients who received no Amigen (see line charts at bottom of Figure 2).

A note of caution is in order about the use of Amigen injections as supplements to diet. Of the 14 patients who received injections, 10 developed anorexia, with or without nausea and vomiting, on 29 out of 48 occasions, although the rate of injection never exceeded 6 grams of Amigen in 2 hours and was usually slower than this. Loss of appetite, the most prominent symptom, prevented patients from eating their full meals during and immediately after infusions. It was, therefore, found advisable to give the Amigen supplements at the end of the day, some time after the evening meal, so that if the patients vomited they sacrificed little food. Since the injection of 12 grams of Amigen lasted 4 hours or more, this interfered with rest and sleep.

Except when Amigen was given, calorie- and

nitrogen in the diets were roughly proportional to one another. It follows that wastage of nitrogen was no more influenced by caloric intake than by dietary protein.

It cannot be inferred from these experiments that losses of nitrogen could not have been prevented if the intake had been increased still further by injections of larger quantities of Amigen. It was not the purpose of the investigation to examine this point. It has been established that patients with acute infections, injuries, and operations of moderate severity waste nitrogen in their urine when they are given as much protein as is feasible by ordinary means. Had it not been for the special attentions of an excellent team of nurses, it would have been impossible to give the patients the high Calories and protein which they received. Results with the Amigen supplements merely serve to show that even greater efficiency in the introduction of food might have been quite as unsuccessful in preventing wasting.

Neither total serum protein nor serum albumin were significantly altered by the losses of protein sustained by these patients. Evidently, either the nature or the degree of malnutrition from which they suffered was such that it did not affect serum albumin. The amino acids of the plasma, which are not given in the table, were also unaffected.

The experiments throw no light on the causes of the obligatory destruction of protein. The conditions under which it is encountered are not those which are generally considered to be associated with great destruction of tissue. Convalescence from meningitis or scarlet fever, for example, would not fall into this category. In any case, there is no reason why the products of autolysis of tissue should not be as nutritious as products of digestion or protein hydrolysates made in the laboratory. Conversely, if the organism is unable to avail itself of autolytic products, it is not altogether surprising that it does not utilize hydrolysates or products of digestion. The old concept that there are two kinds of protein in the body, a more expendable deposit protein and the tissue proteins, might explain the difference in reactions to chronic and acute injuries and infections and possibly the preservation of serum albumin in these acute conditions. There is no obvious reason, however, why deposit protein should be less

easily replaced than other protein by foods that can maintain normal nutrition and nitrogen equilibrium. It may be that some particular element of protein is expended more rapidly in the acute stages of disease. A few analyses have confirmed the observation of others that the extra urinary nitrogen is chiefly urea. This does not exclude an aberration of metabolism. Urea is the chief excretory product in the urine of animals which receive diets deficient in essential amino acids. Again it is hard to understand, however, why a similar infectious process or injury should have different effects on sound and malnourished subjects.

Because it is not possible to prevent subjects with acute infections or injuries from wasting protein by the administration of generous diets, it does not follow that efforts to feed such patients adequately should be abandoned, unless it can be shown that generous diets are positively injurious and that the nitrogen wastage is a beneficial process. After ordinary operations, negative nitrogen balances are short-lived; their occurrence and duration in acute infections are unpredictable. Besides, there is a general clinical impression that the well-being of patients is favored and convalescence accelerated by liberal diets.

This investigation has demonstrated that patients so sick that they would eat little if left to their own devices and the usual services of a hospital ward can, by assiduous nursing care, usually be induced to take 90 to 125 grams of protein and 2500 to 3500 Calories or even more daily without resort to parenteral administration of Amigen.

CONCLUSION

Patients with acute infectious diseases and following some operations were found to have negative nitrogen balances in spite of high caloric and high protein intakes. Further increases of nitrogen intake with parenteral casein hydrolysate failed to affect nitrogen equilibrium except in a few instances.

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ANTISTREPTOLYSIN TITERS IN CASES OF FILARIASIS WITH RECURRENT LYMPHANGITIS AMONG MILITARY PERSONNEL

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Since the outbreak of the war, thousands of military personnel have been stationed in islands of the South Pacific where a non-periodic type of Bancroftian filariasis is prevalent among the native populations, and large numbers of the troops, who have been intensively exposed to the bites of infected mosquitoes, have developed clinical manifestations of filariasis. The pattern of the disease in these men is similar to that described many years ago (1) in Europeans, a few months after their arrival in these same island groups, and consists primarily of a lymphadenitis and remittent lymphangitis, sometimes associated with constitutional symptoms of low-grade fever, chills, malaise, and nausea. The natives are well acquainted with this syndrome and call it "mumu."

The chief manifestations of "mumu," as seen among the troops, are lymphadenopathy of the axillary, inguinal, and epitrochlear nodes, with recurrent attacks of a retrograde or centrifugal lymphangitis, and the appearance of evanescent red, swollen, pruritic areas on the affected trunk or extremities (2, 3). The spermatic cord and scrotal contents are frequently affected, the commonest lesion being a funiculitis, with or without epididymitis, orchitis, or hydrocele (4). In the majority of cases, a definitive diagnosis of filariasis cannot be made, since microfilariae have thus far been found but rarely in the blood (3, 5); but the adult parasites have been recovered from involved lymph nodes in a number of patients (6, 7), and skin tests with filarial antigens have yielded positive results in about 85 per cent of the men with clinical evidence of the disease (2, 3, 6, 8).

The mechanism which excites the attacks of lymphangitis and fugitive swellings in these cases is of great interest. The aforementioned observers are in general agreement that the chief factor is a sensitization of the host to antigenic sub-

stances elaborated by the adult parasites, and that the symptoms are the result of an immune or allergic type of reaction. This opinion is supported by the high incidence of positive skin tests, precipitin tests, and complement fixation reactions with filarial antigens among supposedly infected troops (2, 3, 6, 8), indicating that the majority have developed antibodies to the parasite; by the microscopic appearance of the tissue changes in biopsy specimens (6, 7); and by the fact that the local reactions occurring spontaneously in these naturally infected individuals may be reproduced in many of them following the intradermal inoculation of filarial antigens (2).

Another possible mechanism for the recurrent lymphangitis is secondary bacterial infection, since it is well-known from earlier work (9) that lymph stasis and lymph edema predispose to bacterial invasion, particularly with hemolytic streptococci. Furthermore, the so-called "tropical lymphangitis" of the Caribbean area, which clinically resembles the lymphangitis of "mumu" in some respects, has been definitely shown by bacteriological and serological procedures frequently to be initiated by streptococcal infections (10).

The infectious etiology of the lymphangitis seen in the cases of filariasis among military personnel has been investigated, using ordinary bacteriological procedures, and thus far cultures have failed to reveal hemolytic streptococci (6, 7). Cultural methods alone may, however, be inadequate, as was clearly demonstrated in cases of tropical lymphangitis (11), in which it was found to be difficult to isolate streptococci at the time of the attacks. It was shown, nevertheless, that the attacks were engendered by a streptococcal infection, since the majority of these cases subsequently showed an immune response characterized by an augmented titer of antistreptolysin "O" in the blood.

The present report deals with an investigation of antistreptolysin titers in the blood of soldiers suffering from recurrent lymphangitis associated with filariasis. The study was undertaken to obtain additional evidence for or against streptococcal infections as a factor in producing the symptoms exhibited by these men.

CLINICAL MATERIAL AND GENERAL PROCEDURES

The clinical material consisted of 45 cases of suspected filariasis among military personnel recently returned from the South Pacific area, most of whom were observed by the authors at the Halloran General Hospital, Staten Island, New York.¹ The remaining cases were seen at the Harmon General Hospital, Longview, Texas, by Colonel Alexander Marble and Captain A. A. Goodman, through whose interest and cooperation the clinical data and blood specimens were obtained.

All of the cases gave a history of one or more attacks of lymphangitis beginning 5 to 12 months after their initial exposure to infection, and the majority had regional lymphadenopathy at the time of examination. Many of the men had also noted transient painful swellings of the spermatic cord or scrotum, although only a few showed persistent enlargement of these structures. None of the cases gave a history of recent sore throats or other infections of the respiratory tract, nor of infections following injury to the body surface, but nearly all had had an attack of lymphangitis within 2 months preceding the date when blood was drawn for determination of the antistreptolysin titer.

Antistreptolysin titers were determined on single specimens of blood from each case; multiple specimens could not be obtained owing to the early transfer of the patients to other areas. The tests were performed by a modification of the method described by Coburn and Pauli (12), the final titers being reported as the highest dilutions of inactivated sera that would completely neutralize one standard unit of streptolysin (Todd).

Each patient was also skin-tested with filarial antigen prepared from *Litomosoides carinii*, as described in a previous publication (8). One tenth ml. of this antigen, in a dilution of 1-200, was injected intracutaneously on the volar surface of one forearm, together with a similar amount of the diluent alone on the other forearm as a control. Positive reactions were characterized by the almost immediate appearance of an urticarial wheal at the site of the injected antigen, accompanied by surrounding erythema and usually by some local itching. The intensity of the reactions was estimated by measuring the longest perpendicular diameters of the wheals, 10 minutes after injection.

Blood smears to be examined for microfilariae were obtained by preparing thick films as for malarial parasites,

de-hemoglobinizing in tap water, fixing in equal parts of ether and absolute alcohol, and staining with hematoxylin.

RESULTS

Antistreptolysin titers. The results of the tests for antistreptolysin titers are recorded in Table I.

TABLE I
Distribution of antistreptolysin titers in 45 cases of filariasis among military personnel

Antistreptolysin titer	Number of cases	Antistreptolysin titer	Number of cases
20	2	125	2
33	4	142	7
62	4	166	4
71	4	200	3
83	5	250	3
100	2	333	3
111	2		

It will be seen that the titers varied from 20 to 333 units per ml. of serum; but, of the 45 cases, only 6 were found to have titers of 250 or more, while the remaining 39 cases showed values of 200 or less. It is the opinion of most observers that the range of antistreptolysin titers which may be regarded as normal is variable; however, for single specimens, it is generally considered that a titer of 250 units or more is elevated, while a titer lower than this is within normal limits (13). According to these criteria, therefore, the great majority of the cases of filariasis failed to show serological evidence of an antecedent streptococcal infection.

It is of interest further to compare the antistreptolysin titers obtained in this group of 45 cases with those found by others (14) among 47 normal male medical students. The figures for the two groups are given in Table II and show an

TABLE II
Comparison of antistreptolysin titers in 45 cases of filariasis and 47 normal adult males

Antistreptolysin titers	45 cases of filariasis	47 normal males (Rantz, Kirby and Jacob)
Titer 100 or less	21	23
Titer 101 to 166	15	18
Titer 200	3	
Titer 250+	6	6

extremely close correlation, which is evidence that the distribution of the antistreptolysin titers among the cases of filariasis is similar to that

¹ These cases were made available for study through the kindness of Colonel Ralph G. De Voe, Commanding Officer of Halloran General Hospital.

TABLE III

Antistreptolysin titers in a case of tropical lymphangitis initiated by a hemolytic streptococcal infection

	Interval after attack of lymphangitis						
	2 weeks	3 weeks	4 weeks	5 weeks	8 weeks	13 weeks	23 weeks
Antistreptolysin titer	550	550	550	620	830	550	500

which may be found in normal males of approximately the same age.

In Table III, serial determinations of the antistreptolysin titer in a patient from the Caribbean area who suffered a typical attack of "tropical lymphangitis" are recorded. It will be noted that the antistreptolysin titer, which was 550 units 2 weeks after the attack, was still elevated at the twenty-third week, when the tests were discontinued. The high titers in this case illustrate the type of response which led other workers (11) to the conclusion that most cases of tropical lymphangitis are engendered by an infection with hemolytic streptococci; they are in marked contrast to the normal titers encountered in nearly all of the cases in our present series.

Skin tests. The skin tests with filarial antigen prepared from *L. carinii* were positive in 38 of the 45 cases, an incidence of 84.5 per cent. These results are almost identical with those obtained (2, 6) in a large series of cases tested with *Dirofilaria immitis* antigen, and serve to indicate that the majority of the men had serological as well as clinical evidence of a filarial infection.

No correlation was observed between the size of the skin wheals and the levels of the antistreptolysin titers, as is shown in Table IV. However, it was noted that the men who had suffered most severely from recurrent lymphangitis were, in a number of instances, those who displayed the most marked cutaneous responses to filarial antigen.

Microfilariae in the blood. Microfilariae were detected in the blood of only one patient. This finding is in harmony with that of the other investigators who have studied recently acquired filariasis among our military personnel (2, 3, 5, 6).

DISCUSSION

Although a definitive diagnosis of filariasis was made in only one patient of the present series by the discovery of microfilariae in the peripheral

blood, there is good indirect evidence that the great majority, if not all, of the men, were suffering from a filarial infection contracted during their residence in areas of the South Pacific where the disease is highly endemic. Their symptoms, particularly the lymphadenopathy and recurrent lymphangitis, strongly suggested such an infection, and it is especially significant, from the diagnostic standpoint, that a large proportion of the men had developed a demonstrable immunological reaction to an antigen prepared from a filarial worm which is closely related to *Wuchereria bancrofti*.

The antistreptolysin titers were within normal limits in nearly all of these cases, only 6 of the 45 showing titers which were high enough to suggest the possibility of a recent streptococcal infection. These findings are in contrast to those of Morales and Pomales among cases of so-called "tropical

TABLE IV

Comparison of intensity of cutaneous reactions to filarial antigen and level of antistreptolysin titers in 45 cases of filariasis

Case	Skin tests: diameters of wheals	Antistreptolysin titers	Case	Skin tests: diameters of wheals	Antistreptolysin titers
	cm.			cm.	
1	1.1 × 1.1	71	24	1.9 × 1.8	100
2	1.2 × 1.0	62	25	2.0 × 1.8	111
3	1.2 × 1.0	83	26	2.0 × 2.0	83
4	1.2 × 1.1	33	27	2.1 × 1.7	333
5	1.2 × 1.1	200	28	2.1 × 2.0	166
6	1.3 × 1.0	33	29	2.2 × 2.0	83
7	1.3 × 1.1	142	30	2.2 × 2.0	71
8	1.4 × 1.4	142	31	2.2 × 2.0	200
9	1.5 × 1.2	166	32	2.4 × 1.8	71
10	1.5 × 1.3	250	33	2.4 × 2.0	62
11	1.5 × 1.5	33	34	2.5 × 2.3	83
12	1.7 × 1.5	62	35	2.5 × 2.4	333
13	1.7 × 1.6	125	36	2.6 × 2.2	166
14	1.7 × 1.7	250	37	3.3 × 2.5	200
15	1.7 × 1.7	142	38	Positive	20
16	1.8 × 1.5	166	39	Negative	250
17	1.8 × 1.5	142	40	Negative	333
18	1.8 × 1.6	33	41	Negative	20
19	1.8 × 1.6	142	42	Negative	111
20	1.8 × 1.7	62	43	Negative	142
21	1.8 × 1.8	100	44	Negative	83
22	1.9 × 1.7	71	45	Negative	125
23	1.9 × 1.7	142			

lymphangitis," most of whom showed distinctly elevated levels of antistreptolysin in the blood following attacks of the disease. It seems only reasonable to assume, therefore, that while a secondary streptococcal infection may possibly have initiated the lymphangitis in a few of our cases, the responsible factor in the majority was of a different nature.

Unfortunately, our clinical data were not sufficiently complete so that a truly accurate appraisal of the relationship between the severity of the clinical manifestations and the intensity of the cutaneous responses to filarial antigen could be made. We did note, however, that some of the most marked dermal reactions occurred in patients who had had severe attacks of lymphangitis. In one such individual, following the intradermal injection of 0.1 ml. of filarial antigen, a generalized reaction occurred within about 20 minutes. This reaction was characterized by the appearance of a pruritic, urticarial eruption over the chest, back, and abdomen, together with a marked facial edema and some dyspnea.

In several other cases, red streaks were observed to radiate up the arm from the site of antigen injection, followed by transient edema of the neighboring soft parts. These secondary manifestations closely simulated a mild attack of lymphangitis, and it is of interest that they could be provoked by such a small amount of antigen injected to the skin.

The sum of these observations leads us to conclude, as have other investigators, that the recurrent lymphangitis seen in these men can be adequately explained as the result of allergic reactions to the parasitic infection.

SUMMARY

Antistreptolysin titers were determined in the blood of 45 soldiers suffering from recurrent lymphangitis associated with filariasis contracted in the South Pacific. The titers were within normal limits in 39 of the 45 cases.

The attacks of lymphangitis in these soldiers appeared to be due probably to allergic reactions

to the parasitic infection; secondary streptococcal infections were apparently of little or no consequence as an etiological factor.

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PROTECTIVE EFFECT OF VACCINATION AGAINST INDUCED INFLUENZA A¹

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In December 1942, rather extensive studies, which will be reported separately, were begun for the purpose of determining the value of subcutaneous vaccination with a vaccine of inactivated influenza viruses, Types A and B, against the epidemic form of influenza. The anticipated outbreak of this disease did not occur but the opportunity to test the resistance of certain of the vaccinated individuals to induced infection presented itself. This was done by inducing infection with strains of virus maintained under laboratory conditions. Other investigators (1) had previously reported that, by the use of such a procedure, vaccine containing inactivated Type A influenza virus, given subcutaneously, was demonstrated to have protected children against experimental infection.

A preliminary report of the clinical results has already been made (2). The present report is intended to present the details of the evidence for the protective effect of vaccination against induced influenza A, including the laboratory investigations which constituted an important phase of the study.

MATERIALS AND METHODS

Vaccine. The vaccine employed was prepared by the medical research division of Sharp & Dohme³ according to specifications furnished by the Influenza Commission and purchased at a minimal cost with Commission funds.

¹ These investigations were aided through the Commission on Influenza, Board for the Investigation and Control of Influenza and other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army. This study was also aided by a grant from the International Health Division of the Rockefeller Foundation.

² Fellow in the Medical Sciences of the National Research Council 1942-1943.

³ The authors wish to express to Dr. Wm. A. Feirer and Dr. Bettylee Hampil an appreciation of their active interest and cooperation in the preparation of the material.

As a source of virus, 11-day-old embryonated eggs were inoculated with a 10⁻³ dilution of infectious allantoic fluid. The PR8 strain (3) of Type A virus and the Lee strain (4) of Type B virus were used. The virus contained in the harvested extra-embryonic fluids was adsorbed onto the embryonic erythrocytes and concentrated by elution in $\frac{1}{10}$ the original volume of saline (5). The Type A and Type B materials were prepared separately and tested for virus content by agglutination of chicken red cells and also by infectivity tests in mice. The virus was inactivated by the addition of formalin in a final concentration of 1:2000 and phenyl mercuric nitrate, 1:100,000, was added for bacteriostatic purposes. The Types A and B viruses were pooled in equal volumes and after bulk sterility tests were bottled in 50 ml. volumes. The other standard tests as specified by the National Institute of Health for sterility and safety required for biological products were then made.

The immunizing capacity (10) of the vaccine for mice was such that 2 doses of 0.5 ml. each of a 2 × 10⁻² dilution of vaccine, given intraperitoneally, a week apart, protected mice against at least 10,000 50 per cent mortality doses of mouse passage PR8 virus, given intranasally, 1 week after the last intraperitoneal injection.

Virus preparation used for infection. The virus used for inducing infection in the human subjects was the Baum strain of Type A influenza virus isolated in 1940 in ferrets inoculated with throat washings from a patient acutely ill in the epidemic. The strain is similar to but not antigenically identical with the PR8 strain of Type A virus present in the vaccine. Frozen and dried mouse lung tissue, which had been stored for nearly 2 years, served as seed for preparation of the infected allantoic fluid employed for the human infection. The virus had been passed through 9 ferrets and 10 series of mice before egg inoculation. Material from the 3rd egg passage was used. Fluid without red blood cells was aspirated from the allantoic sac of 13-day-old embryos 48 hours after inoculation. The material was clarified by centrifugation, placed in rubber capped vaccine vials, and kept at 4° C. for 20 days before use. Tests for bacterial sterility were made in Brewer's thioglycollate medium, in plain beef heart infusion broth, and by inoculation of 11-day-old chick embryos. At the time the fluid was used for human infection, it had the following properties:

(1) Intranasal infective titer for mice:

10⁻² 10⁻³ 10⁻⁴ 10⁻⁵ 10⁻⁶ 10⁻⁷
 3, 4, 4 3, 5, 5 5, 6, 6 5, 6, 7 ++++, ++, ± +, +, +

(Figures denote day of death of individual mice, and plus signs refer to degree of pulmonary consolidation in survivors autopsied on the 10th day.)

(2) Agglutinating titer for chicken's red cells was 1280 (6, 7).

Subjects. The subjects were 102 male residents of a single ward of the Ypsilanti State Hospital, Ypsilanti, Michigan. The men ranged in age from 20 to 70 years, but the majority were between the ages of 30 and 54 years. For the most part, they were able to give adequate descriptions of their symptoms. They were physically active and almost all were productively employed in the various maintenance services of the institution. When not at their jobs, the men mingled in a large, modern, well ventilated day room and at night were quartered in spacious dormitories containing 20 to 30 beds each with space of approximately 3 feet between beds; a few men had individual rooms. They took their meals in a cafeteria together with men from other wards.

Vaccination. On December 21, 1942, alternate residents of the ward received a subcutaneous injection of 1 ml. of the combined Types A and B vaccine. In the same manner and at the same time, the others were given 1 ml. of physiological salt solution containing formalin and preservative in quantities similar to that present in the vaccine. The subjects did not know which material they had received. Careful daily observations were made during the subsequent 4½ months during which time no evidence of influenza A was found.

On April 21, 1943, the ward population comprised 45 individuals who had received vaccine in December 1942, and 57 who were either uninoculated or were given an inoculation of control material. At this time, 4 months after the initial vaccination, 17 of the 45 subjects in the vaccinated group were given a second inoculation of the same lot of vaccine which had been administered in December. At the same time, 21 of the 57 in the unvaccinated group received an initial injection of vaccine. The vaccine had been stored at 4° C. since preparation.

Infection. On May 4, 1943, all subjects were exposed in a uniform manner to influenza virus Type A. Divided according to prior treatment, the ward population consisted of the following groups:

- (1) A control group of 36 unvaccinated individuals;
- (2) 28 men who had received vaccine 4½ months previously;
- (3) 17 men who were twice vaccinated, both 4½ months and 2 weeks prior to infection;
- (4) 21 individuals who had received vaccine 2 weeks before infection.

All subjects were kept from their usual occupations for 24 hours following exposure to the virus; however, as a group they visited the cafeteria for their meals. Patients from other wards used the same dining hall at the same time, but intermingling of groups was avoided. After the first day, all except those who were febrile were allowed to continue regular activity and to take meals in

the cafeteria. Although quarantine precautions were not rigid, no evidence of influenza was observed at this time in any other group within the institution.

The method for introducing the virus, using nebulizers⁴ for dispersing the virus as a very fine spray, was the same as that previously described (8). The spray was directed by means of an adapter into each nostril for 2 minutes and the subject allowed to breathe the mist. Under a pressure of 10 pounds of air, the nebulizers delivered approximately 0.5 ml. of fluid during the 4 minutes' exposure. The procedure was carried out on the ward in a treatment room directly off the corridor leading to the dormitories and day room.

Since there was a free interchange of air between all rooms, virus which escaped into the atmosphere during the course of the spraying was widely disseminated. This was demonstrated by the isolation of virus from the lungs of mice exposed at several locations in the quarters. While the inhalation procedure was in progress, groups of 10 mice in wire-mesh cages were placed in the treatment room as well as in two other locations on the ward, about 25 to 30 feet away. Four days after exposure, 5 mice in each group were given an intranasal inoculation of sterile broth to accentuate the effect of virus that might be present in their lungs (9). The fate of these mice was as follows:

Location	Exposed during spraying	Exposed during spraying and inoculated intranasally with sterile broth 4 days later
Treatment room	6, 6, 6, 7, 9	6, 6, 6, 7, 7
Corridor	x +, +, +, +, ±	7, 7, 7, 9, 9
Day room	+, 0, 0, 0, 0	7, 9, 0, 0, 0

(Numerals denote day of death after exposure. Symbols 0 to +++++ indicate degree of pulmonary involvement in survivors autopsied on 10th day.)
 x only 4 mice in this group.

It is seen that virus was present in sufficient concentration in the atmosphere in different locations of the ward to infect mice exposed for several hours.

Twenty-four hours after the spraying, 10 mice were exposed in the treatment room for 30 minutes, and then treated as above. No pulmonary lesions were observed.

Clinical observations. All subjects were examined for evidence of respiratory disease on the day before exposure to the experimental infection. Sublingual temperatures were taken for 5 minutes at that time and again immediately before inhalation of virus. Subsequently, all were observed twice daily by physicians and nurses. Temperatures, symptoms, and signs of illness were recorded. Observers did not know to which group the individual subjects belonged.

White blood cell counts were obtained from 21 of the subjects on the day before infection and then daily for 4 days.

Röntgenograms of the chests of 3 individuals who had

⁴ Nebulizers for use in administering epinephrin were made by G. E. Miller, Philadelphia, Pennsylvania.

the most pronounced symptoms and fever were taken on the 3rd day of illness.

Serology. Blood for serological study was obtained from all individuals vaccinated on April 21, 1943. On May 3, 1943, the day before exposure to virus, samples of blood were taken from all subjects for determination of the titer of antibodies in the serum before infection and for estimation of the response to vaccine in the inoculated individuals. Blood was again drawn 2 weeks after inhalation of the active virus.

Antibody was titrated by measuring the capacity of serum to inhibit the agglutination of chicken's erythrocytes by influenza virus. The technique followed is a modification (7) of Hirst's (6) procedure. The sera were titrated against the PR8 strain of influenza virus, Type A. A single pool of antigen was used throughout. Some sera were also tested with the Baum strain which had been used for infection. No important differences in serological responses were noted.

CLINICAL PICTURE OF EXPERIMENTALLY INDUCED INFLUENZA A

The clinical picture of the experimentally induced infection resembled a mild form of the naturally occurring disease. The most common initial symptoms were chilliness, headache, generalized body aches, weakness, and cough associated with substernal soreness. A few individuals complained of slight irritation of the throat, mild nasal congestion, and aching eyes. Dizziness and nausea were each recorded once. Anorexia and insomnia were common. All degrees of severity of illness were observed, varying from the complete syndrome, with a sharp febrile reaction, to mild indisposition associated with definite but slight achiness and headache with little or no fever.

While temperatures of 99° to 99.8° may have reflected a mild illness, these were difficult to evaluate since they were noted irregularly in individuals before infection. Correlation between symptoms and degree of fever revealed that temperatures of 100° F. or more could be considered indicative of distinct clinical disease and a temperature of less than 100° was therefore not considered to be evidence of infection.

In the group of unvaccinated subjects, the highest temperature observed was 103° F. In the majority of those who developed distinct febrile reactions, temperatures of 100° or more were first noted within 24 hours after inhalation; in the remainder, fever began between 24 and 48 hours after exposure to the virus. On the basis of

temperatures taken twice daily, elevations of 100° or more lasted in most instances no longer than 24 hours after onset, in a few persisted for 48 hours, and for as long as 60 hours in only one subject. In no instance in which the maximum temperature recorded was less than 101° did fever last longer than 24 hours. However, the majority of those with temperatures of 101° or more had fever persisting more than 24 hours. No difference was apparent in the features of illness observed in vaccinated and control subjects when cases with corresponding degrees of fever were compared.

Physical examination revealed flushed skin and varying degrees of prostration, usually paralleling the height of fever. Spasmodic, dry cough commonly appeared early. In some, on the second day of illness, slight redness of the pharyngeal and nasal mucosae was evident, occasionally accompanied by nasal congestion. Abnormal physical signs were detected in the chests of 3 of the subjects with the most marked reactions. The findings, over localized areas and involving a major portion of a lobe, were medium, high-pitched, sibilant râles, more marked during the expiratory phase of respiration, together with scattered, fine, moist râles, audible during both inspiration and expiration. Breath sounds were diminished slightly in intensity and were of bronchovesicular quality. No obvious alteration in percussion note accompanied the auscultatory findings. Roentgenograms of the chest on the 3rd day of illness revealed no abnormalities in any of the 3. The abnormal physical signs disappeared within 1 week.

In the most severe cases, a moderate degree of asthenia was present for several days after subsidence of fever, and a slight spasmodic, non-productive cough persisted in many for as long as a week. On the whole, the disease was mild with prompt and uneventful recovery.

White blood cell counts taken on days following inoculation tended to be lower in comparison with the pre-inoculation level. This trend was not uniform nor did it occur to a marked degree.

DISTRIBUTION OF CLINICAL DISEASE IN CONTROL AND VACCINATED GROUPS

Analysis of the clinical responses of the different groups to inhalation of virus revealed dis-

TABLE I

Effect of subcutaneous vaccination upon febrile response of human subjects to induced infection with influenza virus, Type A

Vaccination record	Number of subjects	Highest temperature									
		<99		99 +		100 +		101 +		102 +	
		No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
Unvaccinated	36	7	19	29	81	18	50	9	25	4	11
4½ mos. before	28	8	29	20	71	9	32	3	11	1	4
2 weeks before	21	7	33	14	67	3	14	0	0	0	0
4½ mos. and 2 weeks before	17	6	35	11	65	3	18	0	0	0	0

tinct and significant differences (2). The results have been expressed in terms of the number of subjects reacting with temperatures above the various levels in order to emphasize the observation that among the several groups there is a difference not only in the incidence of temperatures above 100° F., but in the severity of illness as well, as is indicated by the height of the febrile response. Table I summarizes the maximum temperatures recorded. Temperatures of 100° or more were observed in 50 per cent of the controls, in 32 per cent of those vaccinated 4½ months before exposure, in 14 per cent of those vaccinated 2 weeks before, and in 18 per cent of those vaccinated both 4½ months and 2 weeks before; the incidence in the 2 groups, totalling 38 individuals, who had been vaccinated 2 weeks before infection was 16 per cent. Moreover, none of the latter had temperatures higher than 100.8°, while 25 per cent of the control group and 11 per cent of the men vaccinated 4½ months earlier had fevers of 101° or higher. Febrile reactions of 102° or more were observed in 11 per cent of the controls as compared with 4 per cent of the group vaccinated 4½ months before infection.

The immunizing effect seemed to be more marked in the 2 groups vaccinated within 2 weeks of exposure. Individuals receiving 2 inoculations 4½ months and 2 weeks prior to exposure appeared to have had no advantage over those receiving a single inoculation within the shorter interval. The group vaccinated but once, 4½ months before infection, was not as resistant as either of the other 2 vaccinated groups, suggesting that immunity may have waned in the 4½-month interval between vaccination and exposure to infection. It is interesting to note that the distinct clinical reactions in the former group occurred

chiefly among those who had the lower titers of serum antibody.

SEROLOGICAL RESPONSES OF VACCINATED AND REVACCINATED SUBJECTS

Antibody responses were determined in subjects receiving their initial injection of vaccine in April 1943 as well as in those subjects who were reinoculated at that time, 4 months after their first injection. Antibody response to the vaccination done in December 1942 was studied in only 10 per cent of the population vaccinated at that time. Consequently, the number of subjects in the present group who were tested following vaccination are too few to discuss. Since antibody response of human subjects to vaccination varies widely, studies of the antigenic activity of vaccines must be done in large groups of individuals. For this reason, the results obtained in the small groups involved in this experiment can be considered to indicate, only in a general way, the antigenic effect of the vaccine. Studies on larger groups, spread throughout a greater population will be reported subsequently (11).

As shown in Figure 1, 17 of the 21, or 81 per cent, of the group vaccinated for the first time 2 weeks before infection had 2-fold or greater increase in antibody titers and 12, or 57 per cent, had increases in titers of 4-fold or more. In the revaccinated group, 6 of 16 tested, or 38 per cent, had 2-fold or greater increases in titers, and only 2, or 13 per cent, had greater than 2-fold increases. Both of the latter had titers of less than 32 prior to the second injection. As has been observed previously, changes in titers occur less regularly and are of relatively slight degree in individuals who have high antibody titers at the time of injection (12). Revaccination appeared

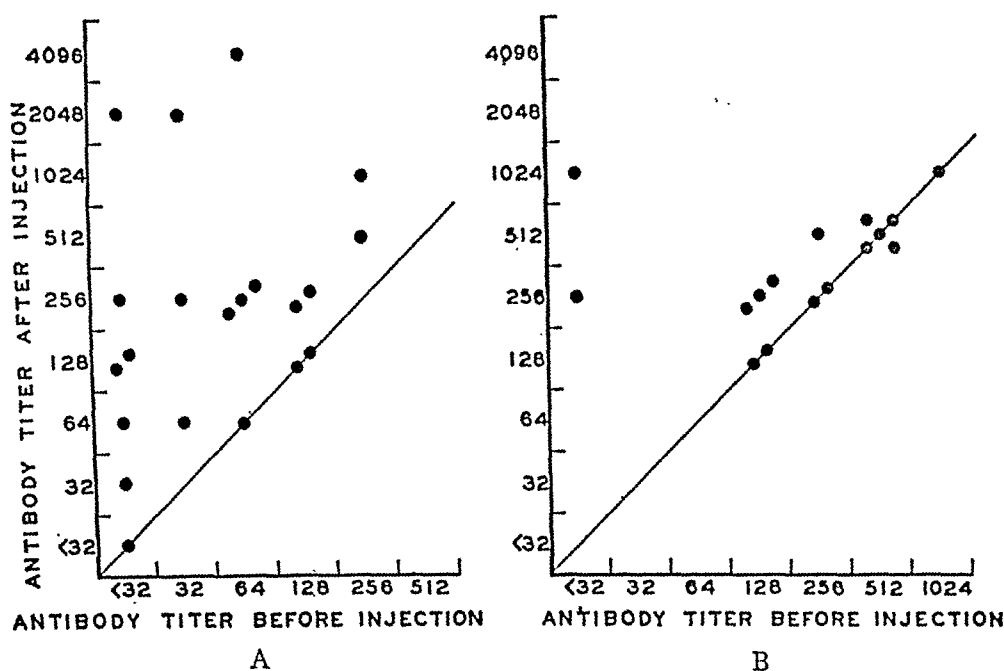


FIG. 1. A. CHANGE IN TYPE A ANTIBODY TITER FOLLOWING FIRST INJECTION OF VACCINE. B. CHANGE IN TYPE A ANTIBODY TITER FOLLOWING SECOND INJECTION OF VACCINE, 4 MONTHS AFTER FIRST

to have had only a slight influence on the antibody levels of the group as a whole. In this respect, reinoculation of a group of adults, who probably have had multiple experiences with influenza viruses, did not seem to have a "booster" effect.

RELATION BETWEEN TITER OF SERUM ANTIBODY AND RESISTANCE TO INFECTION

The data obtained in this experiment were examined to determine whether or not resistance to infection, as measured by febrile reaction, was, to any degree, related to the level of serum antibody. The relationships are shown in Figures 2 and 3.

In general, the data in Figure 2 reveal that in the unvaccinated group antibody titers were in the lower range, while in the vaccinated groups antibody titers were higher. Moreover, the incidence and degree of distinct febrile reactions were greater in unvaccinated subjects as compared with vaccinated individuals. It is readily evident from these results that no striking relationship between antibody level and resistance is apparent in any one of the 4 groups, and that if any relationship does exist, the number of individuals in each group is too small to show it conclusively. It can be pointed out that in the group vaccinated 4½ months before infection, 6 of 9 who developed

distinct febrile responses had antibody titers of 128 or less, while 3 out of 17 who had titers of 256 or greater had distinct fever. On the other hand, of the group vaccinated 2 weeks before infection, there were 8 out of 9 with titers of 128 or less who failed to exhibit a clinical response, while 10 out of 12 with titers of 256 or greater did not respond with a significant degree of fever.

When a composite chart is made, by superimposing all 4 groups, it is seen that those who had febrile reactions tended to have the lower antibody titers. Among those with titers of 256 or more, there were few who had a febrile response. When the incidence of temperatures of 100° or more is determined at each level of serum antibody before infection, distinct differences become evident. Table II summarizes the observations illustrated in Figure 3. Temperatures of 100° or more were noted in 26, or 49 per cent, of 53 individuals having pre-infection antibody titers of 128 or less; in 7, or 19 per cent, of 36 with titers of 256 and 512; and in none of the 13 having titers of 1024 or greater. When a separation is made between those with titers of 128 or less, and 256 or more, dividing the group approximately in half, temperatures of 100° or more were found to have occurred in 49 per cent of subjects in the low antibody group as compared

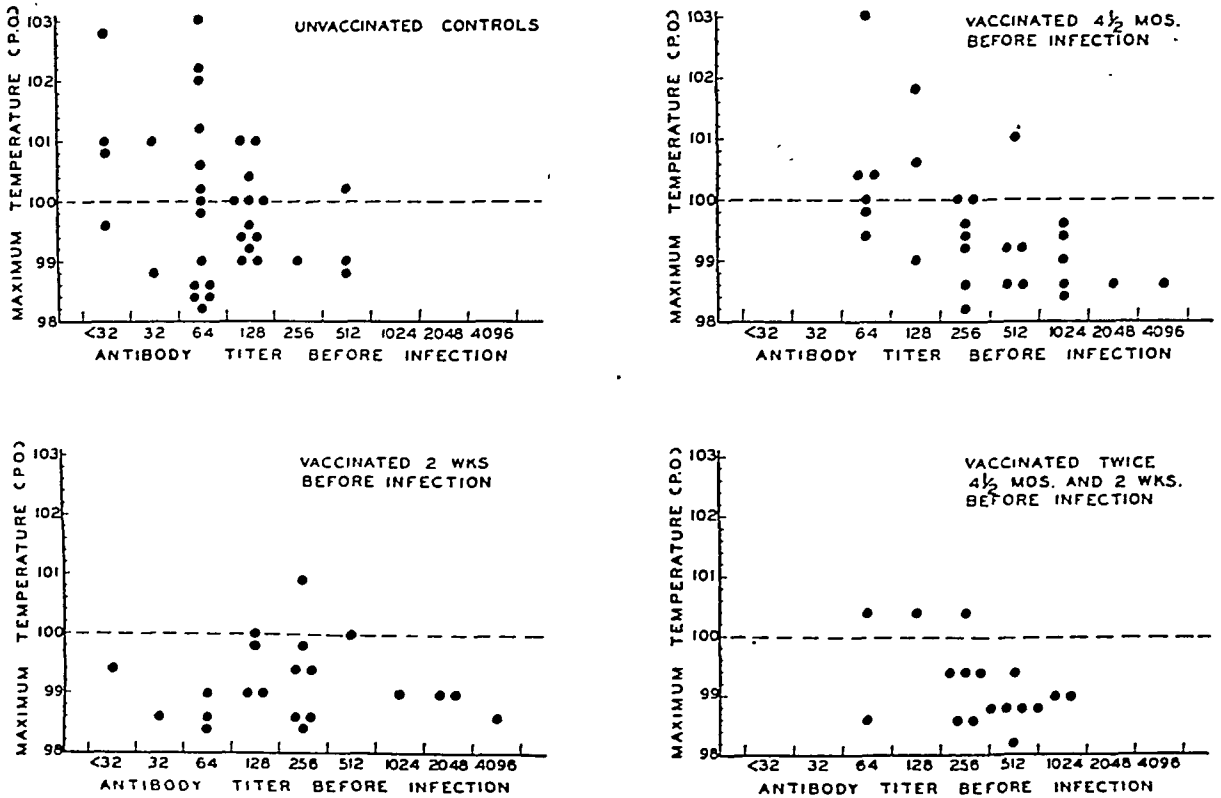


FIG. 2. RELATION OF ANTIBODY TITER AND FEBRILE RESPONSE TO INFLUENZA VIRUS, TYPE A

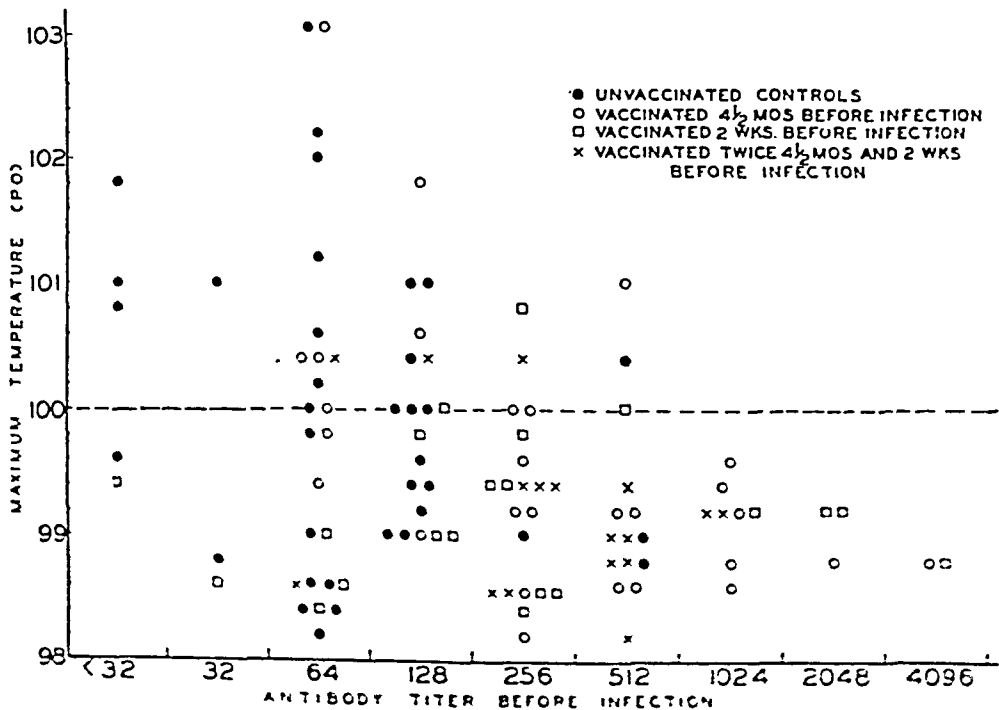


FIG. 3. RELATION OF ANTIBODY TITER AND FEBRILE RESPONSE TO INFLUENZA VIRUS, TYPE A, IN VACCINATED AND CONTROL SUBJECTS

TABLE II

Incidence of febrile reactions of 100° or more in relation to pre-infection antibody titer

Antibody titer	Incidence of temperature of 100° or more					
	No.*	Per cent	No.*	Per cent	No.*	Per cent
32 or less	4/8	50				
64	12/25	48	26/53	49	26/53	49
128	10/20	50				
256	4/21	19	7/36	19		
512	3/15	20			7/49	14
1024 or more	0/13	0	0/13	0		

* Numerator = Number of individuals having temperature of 100° or more.

Denominator = Total number in each group.

with 14 per cent of individuals having the higher levels of antibody. Only one of the latter had as much as 101°, while 11 of the former had 101° or more.

Because of the preponderance of unvaccinated subjects in the low antibody group and of vaccinated individuals in the higher antibody group, as well as the fact that the vaccinated subjects do not represent a uniform group with regard to vaccination experience, the data do not permit a conclusive statement regarding the relationship between antibody level and resistance.

SEROLOGICAL DIAGNOSIS OF INFECTION IN VACCINATED SUBJECTS

In the course of these studies with experimentally induced infections, cases of clinical influenza have been observed both with and without corroborative serological reactions. When the number of cases showing dissociation between clinical reaction, as measured by febrile response, and serological change, are compared in the control and vaccinated groups, distinct differences are evident. Figure 4 relates maximum temperature to antibody response following the inhalation of virus. These results are summarized in Table III.

These data clearly reveal a greater incidence of positive serological reactions following the inhalation of active influenza virus in unvaccinated as compared with previously vaccinated subjects;

TABLE III

Relation of serological response to febrile reaction following inhalation of active virus in control and vaccinated subjects

Antibody response	Temperature reaction							
	100° +				< 100°			
	Controls (18)		Vaccinated (15)		Controls (18)		Vaccinated (61)	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
None	2	11	8	53	10	56	48	79
Two-fold or greater	16	89	7	47	8	44	13	21
Four-fold or greater	13	72	1	7	4	22	2	3

a similar relationship was found when a division was made on the basis of low and high antibody level before infection. This is true both among individuals who exhibited little or no clinical reaction, as measured by temperature, as well as among those showing a distinct clinical response. The fact that antibody rises can occur in the absence of any clinical evidence of infection has been repeatedly noted with the natural disease (13) and with induced infection (1, 8), but the present data emphasize again that clinical infection does not always evoke measurable changes in concentration of serum antibody. The point of significance here is that while a fairly high correlation exists between distinct clinical reactions and positive serological responses in a group of unvaccinated individuals, the correlation between the two was very low in a group of vaccinated subjects. Thus, if antibody response alone were to serve as index of infection, the evidence would be weighted in favor of the vaccinated group.

COMPARISON OF SEROLOGICAL RESPONSE TO VACCINATION AND INFECTION

Serological reactions were compared in 2 groups of subjects one of which received infectious virus by way of the respiratory tract and the other received non-infectious virus by the subcutaneous route. The results are shown in Figure 5. The group used to illustrate the effect of inhalation of active virus is the unvaccinated control group of 36 individuals. Half of the group of 41 illustrating the effect of vaccination is com-

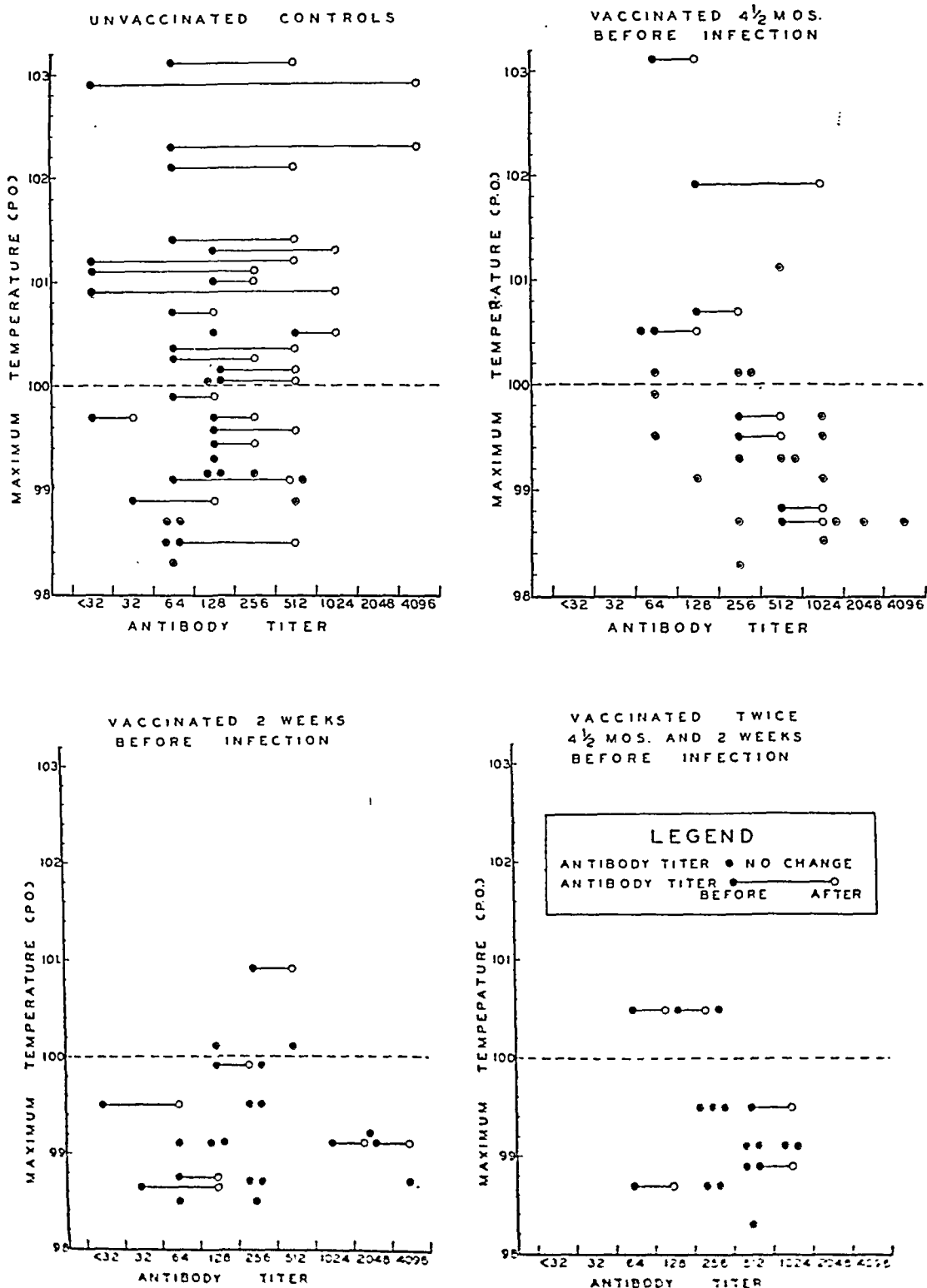


FIG. 4. RELATION OF SEROLOGICAL AND FEBRILE RESPONSES TO INDUCED INFLUENZA TYPE A IN VACCINATED AND CONTROL SUBJECTS

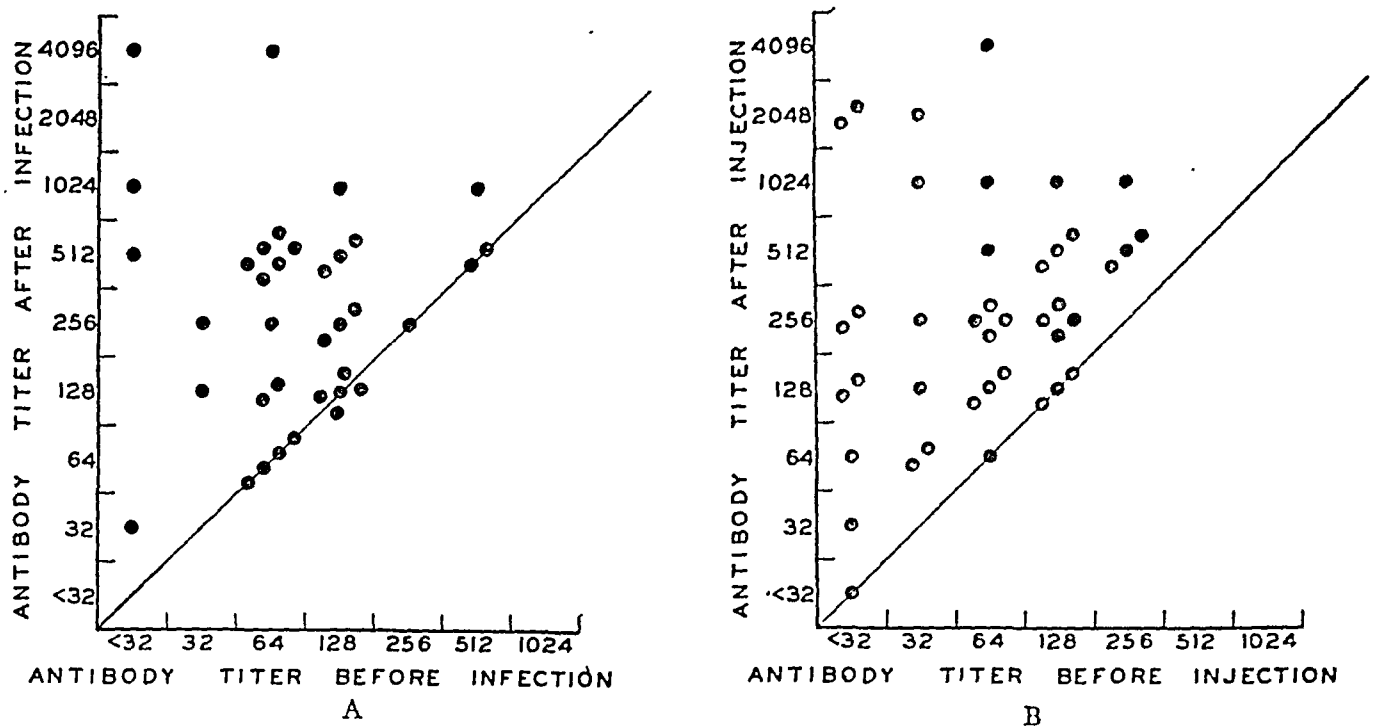


FIG. 5. A. ANTIBODY TITERS BEFORE AND AFTER INHALATION OF ACTIVE INFLUENZA VIRUS, TYPE A.
B. CHANGE IN ANTIBODY TITER FOLLOWING FIRST INJECTION OF VACCINE (TYPE A)

prised of the subjects vaccinated 2 weeks before exposure to the virus and the remainder consists of a group of similarly treated individuals who participated in another study (14). The similarities in response to the administration of virus, in either form, by the different routes was quite striking. The distribution of titers was such that titers of 128 or less were observed in 90 per cent of individuals before vaccination and in 37 per cent after; in the group receiving active virus by inhalation, 89 per cent had titers of 128 or less before treatment and 36 per cent had this level of antibody after treatment.

Four-fold or greater increases in antibody titer were observed in 56 per cent of the vaccinated group and 47 per cent of the group receiving the inhalation; while 2-fold changes in titer occurred in 32 per cent of the vaccinated and 19 per cent of the infected groups. Titers were unchanged in 12 per cent of the vaccinated as compared with 33 per cent of the group treated with active virus. Although approximately the same proportion of subjects reacted with 4-fold or greater increases in antibody titer following either procedure, proportionately fewer individuals failed to show some degree of change in titer after vaccination as compared with infection. The levels to which anti-

body titers were raised were not strikingly different. Similar findings have been reported by others (1).

DISCUSSION

In earlier studies, Francis (10) had shown the importance of the quantitative relationships between the immunizing dose of influenza virus and the resultant immunity in ferrets or mice. Certain investigators (15) have reported that, within limits, proportionately higher antibody levels are obtained when vaccines containing increasing amounts of influenza virus are injected subcutaneously in humans. While others (1) were able to demonstrate the protective effect of subcutaneously injected allantoic fluid vaccine containing influenza virus Type A, against induced infection in children, they noted no difference in the antibody response among groups receiving different amounts of virus. Despite the lack of direct evidence in human individuals, the possibility remained that, with larger amounts of virus in the vaccinating dose, the immunity developed might be more readily demonstrable. Consequently, in the present studies, a concentrated vaccine was used.

Because the results herein reported were ob-

tained by experimental infection, their interpretation in terms of the natural disease must retain certain reservations. Nevertheless, by these means, it has been possible to demonstrate greater resistance to infection with influenza virus, Type A, in groups of vaccinated subjects than in a similar group of unvaccinated controls. The reason why there was a relatively large number of persons who responded with febrile reactions in the group vaccinated 4½ months previously cannot be fully explained. It could represent the results of a failure to develop immunity following vaccination or it could mean that there had been a decrease of an immunity which originally was developed in response to inoculation. However, in view of the fact that the groups vaccinated 2 weeks before infection were more resistant, the increased amount of clinical illness in those vaccinated 4 months before testing suggests that the result was related to a waning of resistance which had been more marked earlier. The question of duration of immunity following vaccination requires further study.

Information regarding the effect of re-inoculation in increasing the titer of circulating antibody and its relation to immunity is limited to the observations upon one group of 16 individuals. This has revealed that in a group re-vaccinated 4 months after their initial inoculation, 6 of 16, or 38 per cent, showed a 2-fold or greater increase in titer, and only 2, or 13 per cent had greater than 2-fold increases. Both the latter had titers less than 32 prior to the second injection. Failure of the second inoculation, given 4 months after the first, to provoke a further rise in antibody in many of the subjects and only a slight rise in others, may be due in part to combination of antigen with antibody, rendering the virus antigenically ineffective. What effect the second inoculation may have upon the persistence of the antibody level is not known but it is clear that the additional inoculation after 4 months did not function as a booster dose.

In the present study of induced influenza, there was an opportunity to observe the variation in serological response in subjects who developed the disease. While a fairly high correlation was found to exist between distinct clinical responses and positive serological reaction in a group of unvaccinated individuals, the correlation between

the two was very low in a group of vaccinated subjects. The reason for this dissociation between clinical and serological response is believed to be related, in part at least, to the existence of relatively high antibody titers before infection in the vaccinated groups. It is again evident in the present study that experimental influenza can actually be induced without a demonstrable increase in serum antibody during convalescence. This observation supports further the notion that a certain number of cases, occurring during outbreaks of the natural disease, without serological response, still represent infections with influenza virus.

Serological response in a group of individuals previously exposed to inhalation of attenuated active virus has been employed as index of infection (16). For the most part, these subjects had relatively high antibody titers after their first exposure, and failure of the group as a whole to respond with a second antibody rise was taken to signify immunity. The results of the present study emphasize the fact that if serological findings, rather than clinical observation, are used as index of infection, the evidence is immediately weighted in favor of the vaccinated or recently infected group. As a result of this study of the effectiveness of vaccination against experimentally induced influenza A, it has become apparent that laboratory criteria of infection alone are not sufficient to determine the effectiveness of vaccination against the natural disease.

SUMMARY

The protective effect of a vaccine containing inactivated influenza viruses Types A and B against induced infection with the Type A virus has been studied in man. Distinct clinical reactions, evidenced by temperatures of 100° or more, were observed in 50 per cent of the controls, 32 per cent of those vaccinated 4½ months before, 14 per cent of those vaccinated 2 weeks before, and 18 per cent of those vaccinated twice. Moreover, none in the latter two groups had temperatures higher than 100.8°, while 25 per cent of the controls and 11 per cent of the group vaccinated 4½ months before had fever of 101° or higher.

Distinct febrile reactions, following exposure to

virus, occurred in 49 per cent of 53 individuals having pre-infection antibody titers of 128 or less and in only 14 per cent of those with titers of 256 or more. Moreover, only one of the latter had as much as 101°, while 11 of the former had 101° or more.

Clinical signs of infection with influenza virus may occur with or without serological evidence; the latter set of circumstances is most commonly noted in vaccinated individuals with high antibody titers. Similarly, serological evidence of infection may be obtained with or without obvious clinical signs.

It was noted that a second inoculation of vaccine, 4 months after the first, did not elicit a further sharp rise in antibodies.

The question of duration of immunity following vaccination has been discussed.

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PROTECTIVE EFFECT OF VACCINATION AGAINST INDUCED INFLUENZA B¹

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In the previous paper (1), evidence was presented to show that subcutaneous vaccination of human subjects with a vaccine containing inactivated influenza viruses, Types A and B, was followed by an increased resistance to experimentally induced infection with influenza virus, Type A. The present report deals with the clinical and laboratory results of a similar study illustrating the effect of subcutaneous vaccination upon resistance of another group of individuals to induced infection with influenza virus, Type B.

10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷
3, 4, 4	3, 4, 4	4, 4, 5	5, 6, +++	6, 7, +++	++++, +++, +++

(Figures denote day of death of individual mice. Plus signs refer to degree of pulmonary consolidation in survivors autopsied on the 11th day.)

A brief summary of the clinical results has been reported (2).

MATERIALS AND METHODS

Vaccine. The vaccine employed was the same as that used in the preceding study (1) and contained influenza virus of both Types A and B. The immunizing capacity of the vaccine for mice was such that 2 doses of 0.5 ml. each of a 2×10^{-4} dilution of vaccine, given intraperitoneally a week apart, protected mice against at least 1,000 50 per cent mortality doses of mouse passage Lee virus, given intranasally, 1 week after the last intraperitoneal injection.

Virus preparation used for infection. The Lee strain of Type B influenza virus was used. Virus contained in frozen and dried lung tissue of mice infected with this strain was transferred to eggs for preparation of the in-

fecting allantoic fluid that was employed for the human infection. The mouse lung tissue consisted of a pool of lungs containing virus which had been passed 1 to 5 times in mice, after 9 to 12 passages in ferrets. Allantoic fluid from the second and third egg transfers was concentrated approximately 10-fold by adsorption onto red blood cells of the embryo and elution into physiological salt solution (3). Fluid containing the virus was placed in a rubber-capped vaccine vial and stored at 4° C. for one week before use. Tests for sterility were made. At the time the fluid was used to infect humans it had the following properties:

(1) Infectivity for mice:

(2) The hemagglutination titer was 10, 240 (4, 5).

Subjects. The subjects were similar to those described in the preceding paper (1) and included 96 men residing in a single ward of the Ypsilanti State Hospital, Ypsilanti, Michigan.

Vaccination. On December 21, 1942, a subcutaneous injection of 1 ml. of the combined Types A and B vaccine was given to each patient in half of the ward population. The others were given physiological salt solution containing formalin and preservative in the quantities present in the vaccine.

During the subsequent 4½ months, all cases of respiratory disease resembling influenza were studied. Although a few sporadic cases of Type B influenza were observed in some of the women's wards in March, 1943, no evidence of Type B infection was found in the male side of the institution.

On April 13, 1943, approximately 4 months after the initial inoculations, the residents of the ward comprised 46 vaccinated persons and 50 unvaccinated controls. At this time, 19 of the vaccinated group were given a second injection of the same lot of vaccine administered in December; 23 of the control group received an initial injection of vaccine.

Infection. Two weeks before exposure to infection by inhalation of the Lee strain of Type B virus, the subjects involved in the present experiment inhaled for 1 minute allantoic fluid containing the Baum strain of Type

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² Fellow in the Medical Sciences of the National Research Council 1942-1943.

A virus. The experiment was unsatisfactory since only a few febrile reactions were observed after this brief period of inhalation. Accordingly, the Type A experiment (1) was repeated on an adjacent ward and the present group was used in the test with the antigenically unrelated Type B virus.

On May 10, 1943, all subjects were exposed to infection with the Lee strain of influenza virus Type B. At this time, the ward population was comprised of the following groups:

- (1) A control group of 27 unvaccinated individuals;
- (2) 27 men who had received vaccine 4½ months previously;
- (3) 23 individuals who had received vaccine 4 weeks before infection;
- (4) 19 men who were vaccinated both 4½ months and 4 weeks prior to infection.

The methods of inducing infection and of making observations were essentially the same as described in the previous study. The only difference was in the duration of exposure. In the present experiment, approximately 0.6 ml. of fluid containing virus was inhaled during a period of 5 minutes. Virus was distributed in the atmosphere during the spraying procedure and persisted in the ward for at least 24 hours; virus was detected by exposure of mice in the manner described in the previous report (1).

Throughout the interval of 7 hours during which the virus inhalation was being administered, groups of 10 mice in wire mesh cages were kept in the treatment room and in two other locations on the ward, 25 to 30 feet away. On the following day, another cage of 10 mice was placed on the floor of the corridor just outside the treatment room for a period of 2 hours. Half the mice in each group were given an intranasal inhalation of sterile broth 4 days after exposure of the original groups. All were observed for 11 days, at which time survivors were autopsied for evidence of pulmonary involvement. The results were as follows:

Location	Exposed only	Exposed and inoculated intranasally with sterile broth
Treatment room	7, 7, 7, 8, 9	6, 7, 7, 7, 8
Corridor	9, +++, +++, ++, ++	7, 7, 7, 7, 8
Day room	++, 0, 0, 0, 0	7, 8, ±, 0, 0
Outside treatment room 24 hours later	0, 0, 0, 0, 0	++++, +++, +++, +++, +++, +++, +

(Numerals denote day of death after exposure. Symbols indicate degree of pulmonary involvement in survivors autopsied on 11th day.)

Apparently active virus persisted in the atmosphere or floor dust in the vicinity of the mice exposed 24 hours after the virus was sprayed.

Clinical observations. Before admission to the group all subjects were examined for signs of respiratory disease. Temperatures were taken sublingually prior to and then twice daily for several days following the inhalation of virus. All subjects were seen twice daily by nurses who were familiar with their usual behavior. Observa-

tions were also made by physicians. Observers did not know to which group the subjects belonged.

White blood cell counts were obtained from 21 individuals on the day of exposure to the virus and then daily for 3 days.

Serology. Blood for serological study was obtained from individuals vaccinated on April 13, 1943. On April 27, 1943, samples of blood were taken from all subjects. Titers of antibodies in the sera were determined using the agglutinin-inhibition reaction (4, 5). An interval of 13 days elapsed between the pre-infection bleeding and exposure to the virus. Blood was drawn 2 weeks after inhalation of the active virus.

CLINICAL PICTURE OF EXPERIMENTALLY INDUCED INFLUENZA B

The clinical picture of the experimentally induced Type B infection was similar to that previously described (6). When compared with the illness induced by the Type A virus (1), several differences were evident. In the Type B infection, the incubation period appeared to be shorter, with symptoms and fever beginning less than 20 hours after inhalation of the virus. The illness was milder and of shorter duration than in the infection induced with the Type A virus. In only one subject did a temperature of 100° or greater persist longer than 24 hours after exposure to the virus. The maximal temperature observed was 102.6°. In the Type B infection, respiratory symptoms were usually absent, and in only a few instances was a mild, dry cough observed. Symptoms of chilliness, headache, bodyaches, and mal-

aise generally were parallel to the degree of fever. Recovery was complete in 24 to 48 hours after the inhalation of virus.

Physical signs of infection were few. Flushed skin and some prostration accompanied the fever. Signs of involvement of the respiratory tract were not detected.

DISTRIBUTION OF CLINICAL DISEASE IN CONTROL AND VACCINATED GROUPS

As in the study with influenza A, temperature reactions of 100° or more were considered indicative of distinct clinical response.

The febrile reactions of the vaccinated and control subjects are shown in Table I. In the un-

TABLE I

Effect of subcutaneous vaccination upon febrile response of human subjects to induced infection with influenza virus, Type B

Vaccination record	Number of subjects	Highest temperature									
		<99		99+		100+		101+		102+	
		No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
Unvaccinated	27	5	19	22	81	11	41	6	22	2	7
4½ mos. before	27	12	44	15	56	2	7	0	0	0	0
4 weeks before	23	12	52	11	48	3	13	0	0	0	0
4½ mos. and 4 weeks before	19	4	21	15	79	2	11	0	0	0	0

vaccinated group, 11, or 41 per cent, of the 27 individuals had temperatures of 100° or more; 6, or 22 per cent, had temperatures of 101° or above; and 2, or 7 per cent, had temperatures of 102° or higher. Of the 69 vaccinated subjects, 7, or 10 per cent, had temperatures between 100° and

100.8°, while none had a higher temperature. There was no significant difference in the responses of the groups vaccinated 4½ months before, 4 weeks before, or vaccinated twice before exposure. In contrast to the results obtained in the study with influenza A, there appears to have been no difference in resistance of the group vaccinated 4½ months before, as compared with the groups inoculated within the shorter interval before exposure to infection.

SEROLOGICAL RESPONSES OF VACCINATED AND REVACCINATED SUBJECTS

In this study, the antibody response to the Type B component of the vaccine was determined both in subjects receiving their first injection and in subjects inoculated for the second time. The results shown in Figure 1 were similar to those described for the Type A antigen.

All 21 of the group vaccinated for the first time had a demonstrable increase in serum antibody; 4, or 19 per cent, had a 2-fold change, and 17, or 81 per cent, had a change of 4-fold or more. In the group of 17 who had been vaccinated 4 months earlier, 12, or 70 per cent, had no additional rise in titer following the second inoculation, and the rest showed only a 2-fold change. The distribu-

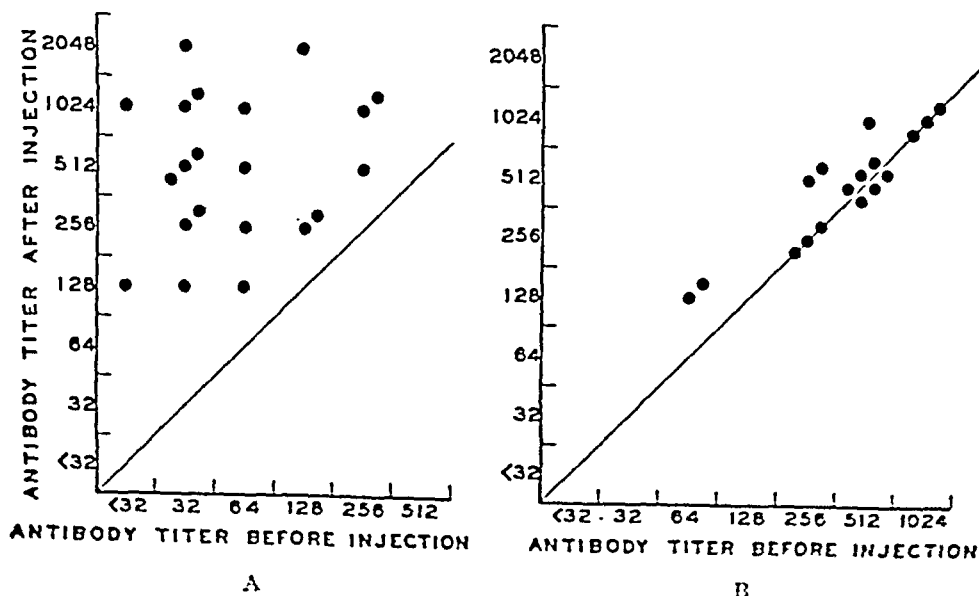


FIG. 1. A. CHANGE IN TYPE B ANTIBODY TITER FOLLOWING FIRST INJECTION OF VACCINE. B. CHANGE IN TYPE B ANTIBODY TITER FOLLOWING SECOND INJECTION OF VACCINE 4 MONTHS AFTER FIRST.

tion of titers before and after vaccination was as follows: In the previously unvaccinated group, 86 per cent had titers of 128 or less before vaccination, while after vaccination only 15 per cent remained within this zone. In the group receiving their second dose of vaccine, 2, or 12 per cent, had titers of 128 or below, both before and after the inoculation. Thus, the effect of the initial inoculation persisted for an interval of 4 months and vaccination at this time exerted little or no effect upon the serological titer. These observations conform with those described in the previous study (1).

RELATION BETWEEN TITER OF SERUM ANTIBODY AND RESISTANCE TO INFECTION

Analysis of the data of this experiment, in order to determine to what extent resistance to induced influenza B may be related to the level of serum antibody, has revealed essentially the same problem that was encountered in the experiment with

influenza A. As shown in Figure 2, the distribution of antibody titers in the control group was predominantly in the lower range, while in the vaccinated groups, titers were clearly higher. Since, in any one group, antibody titers were either in the lower or upper zones, the presence of any relationship between antibody level and resistance would not be evident from an examination of the results observed in any one of the 4 groups. However, from the composite chart, it appears that the incidence and degree of febrile reactions were lower in the group of individuals with antibody titers in the higher zone, as compared with those having lower levels of antibody. Of 58 subjects with antibody titers of 256 or above, only 5, or 9 per cent, had temperatures of 100° or more, while 15, or 35 per cent, of 37 individuals having titers of 128 or less developed fever of 100° or more. Furthermore, only 1 of the former, and 9 of the latter, had temperatures exceeding 100.2°.

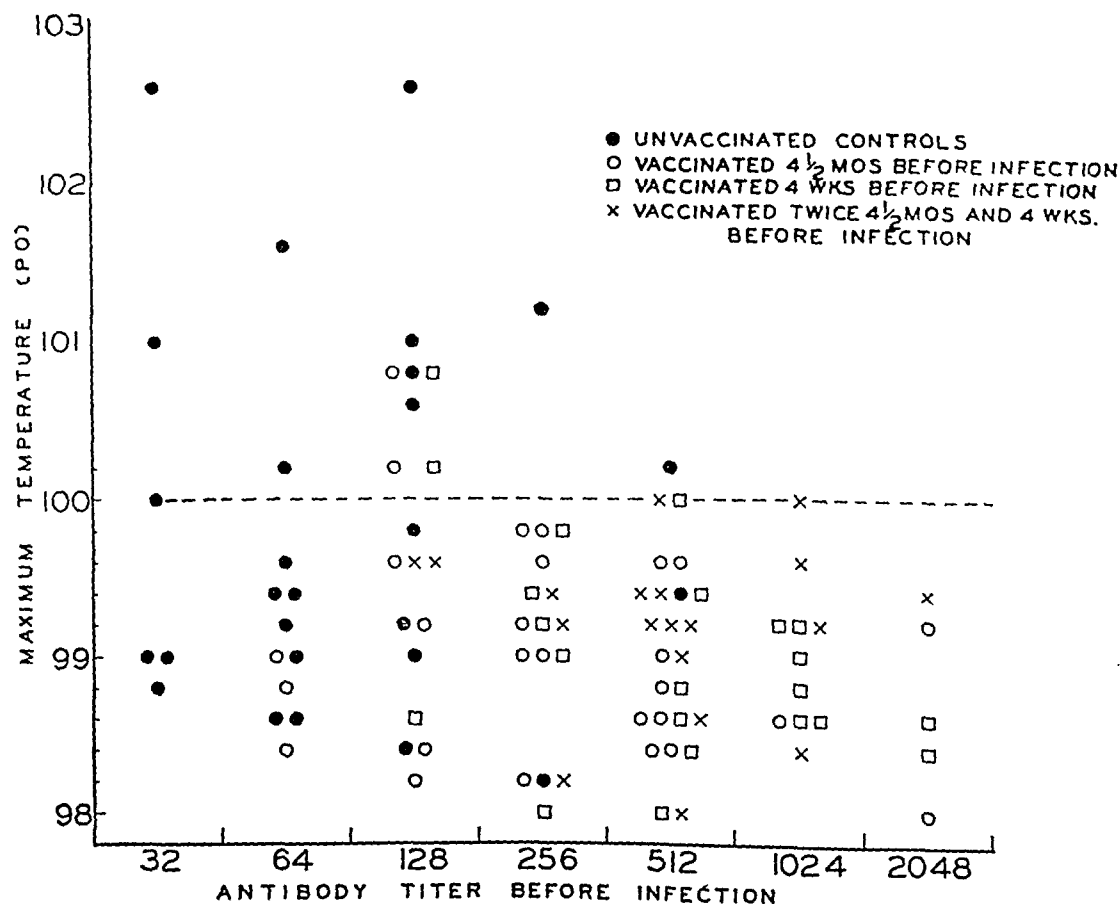


FIG. 2. RELATION OF ANTIBODY TITER AND FEBRILE RESPONSE TO INFLUENZA VIRUS, TYPE B, IN VACCINATED AND CONTROL SUBJECTS

DIAGNOSIS OF INFECTION BY SEROLOGICAL MEANS

In the previous report, it was pointed out that while a fairly high correlation was found between distinct clinical responses and positive serological reactions to inhalation of active influenza virus Type A, in a group of unvaccinated individuals, the correlation between the two was very low in a group of vaccinated subjects.

In the present experiment with a preparation of influenza virus Type B, there were many more instances in which clinical reactions occurred in the absence of serological reactions, both in control and vaccinated subjects. Of the 12 subjects in the control group who had temperatures of 100° or more, 7 had no demonstrable increase in antibody titer; while of the 9 subjects in the vaccinated group who had this degree of fever, 8 had no rise in antibody titer. Among those in the respective groups who did not develop distinct fever, 68 per cent of the controls and 88 per cent of the vaccinated showed no increase in serum antibody.

The reason for the low frequency of serological response in the presence of clear-cut clinical reactions is not apparent. Several pairs of sera were tested by means of the mouse protection test, and the results confirmed the findings of the red cell test.

COMPARISON OF RESISTANCE RESULTING FROM VACCINATION AND INDUCED INFECTION

It is of interest to compare the results of the present study with those reported earlier (6) which were concerned with the response to a second exposure of individuals previously infected with influenza virus, Type B. There were certain differences between the two. In the re-infection study all 23 of the controls reacted with temperatures of 100° or higher and 14 of them had temperatures of 102° or more, while in the control group of the vaccination study, 11 of 27 had temperatures of 100° or more and only 2 of them had temperatures of 102° or higher. Furthermore, in the re-infection experiment, rectal temperatures were recorded, while in the vaccination study temperatures were taken orally.

The results of these two experiments are compared graphically in Figure 3. While temperatures were generally higher in the re-infection ex-

periment as compared with the vaccination study, the relationship between the curves describing the febrile reactions in treated and control groups in both studies was similar. It is probable that the size of the challenge dose in the re-infection experiment had been too great for the forces of resistance in a considerable proportion of individuals who might have resisted a smaller infecting dose. That the previous treatment in each instance had a buffering effect is clearly demonstrated. In fact, it almost seems that the recent infection exerted a greater effect than vaccination, as indicated by the degree of difference between control and treated groups in the respective experiments. While the greater difference between treated and controls in the re-infection study may indicate relatively greater resistance of recently infected subjects as compared with vaccinated individuals, it is equally conceivable that this effect may be related to the degree of multiplication of free virus available for infection, which probably does not vary in simple proportion with the amount of free virus present at the beginning of infection.

DISCUSSION

The results of the present study have clearly demonstrated the beneficial effect of subcutaneous vaccination in preventing infection with influenza virus, Type B, administered intranasally. The effect of the vaccine upon resistance appeared to have persisted for at least 4½ months. It is apparent that, in general, the levels of serum antibody in the vaccinated subjects were higher than in the unvaccinated individuals, and that clinical reactions were, for the most part, fewer and less marked in the former. Nevertheless, it is not possible to conclude from the present data that the beneficial effect of vaccination was due to the heightened concentrations of serum antibody.

Whatever relationship may exist between serum antibody titer and resistance is certainly not an absolute one, as indicated by the observation that many individuals with low levels of serum antibody appear to have resisted infection, whereas some with higher concentrations of serum antibody developed distinct clinical reactions. Because of the superficial site of influenza infection, relatively remote from the direct effect of immune substances in the blood, it is believed that the

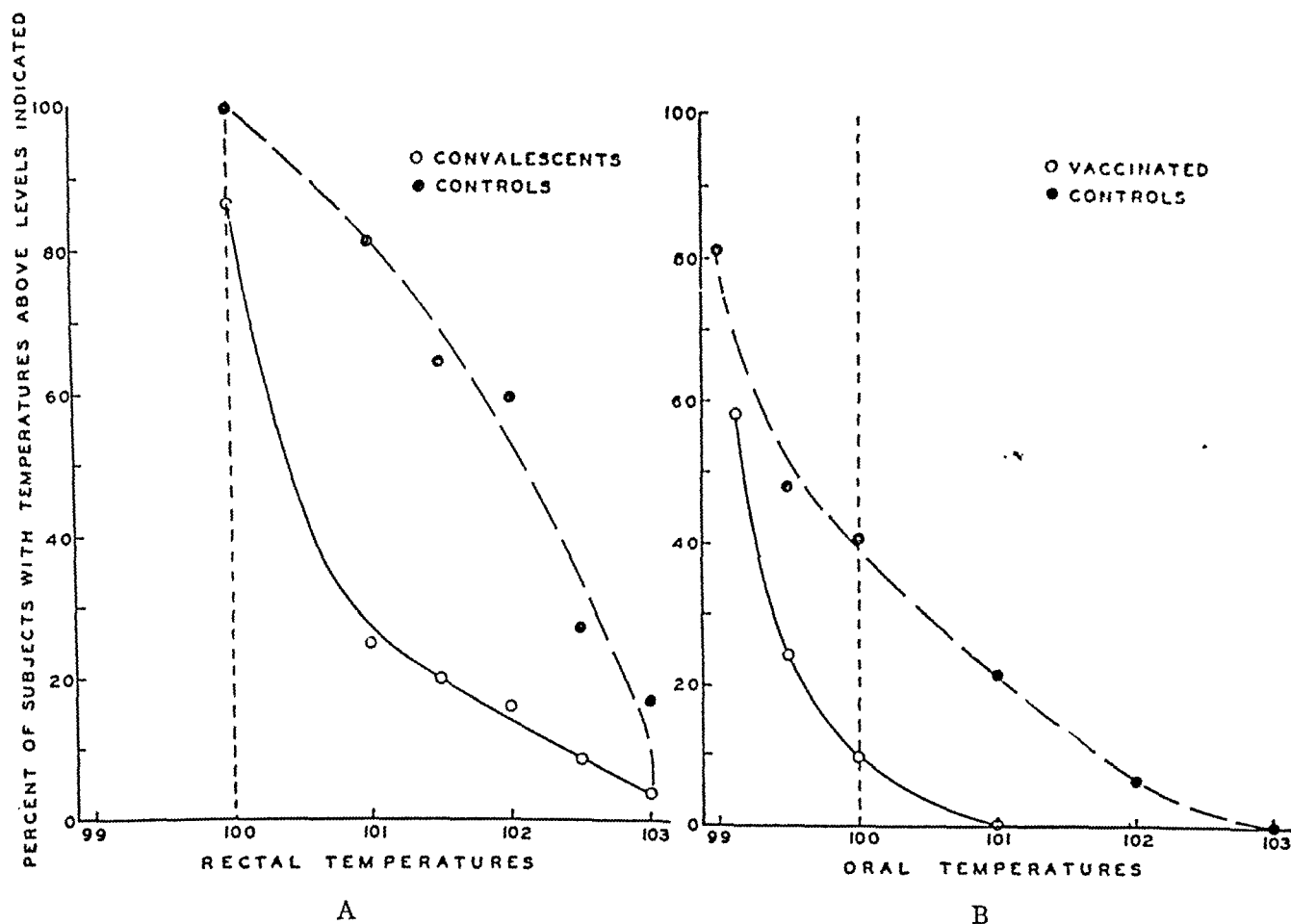


FIG. 3. A. RE-EXPOSURE TO INFLUENZA B, 4 MONTHS AFTER INITIAL INFECTION. B. EXPOSURE TO INFLUENZA B INFECTION FOLLOWING VACCINATION

concentration of antibody in the general circulation cannot be considered more than an indirect index of immunity, and its significance is probably proportional to the effect it may have upon the concentration of antibody in the superficial secretions of the respiratory tract. Physiological features which might affect the interchange of antibody between the two media could under these conditions influence resistance.

The extent to which the mechanism which has been suggested is complemented by cellular modifications, which might result from repeated exposures and increasing age, in contributing to clinical immunity, is not known at the present time. However, recent studies (6) have shown that induced infection with influenza virus, Type B, did not result in uniform resistance to the same strain when tested 4 months later. It is true that the challenge infection was rather severe. Nevertheless, the reaction at the time of the second exposure to virus was not one of complete refractori-

ness, but rather one which might be interpreted as due to a quantitative reduction in the amount of virus available for infection (Figure 3).

SUMMARY

A study was made of the reactions of previously vaccinated individuals to intranasal administration of influenza virus, Type B.

A group of 96 human subjects was divided into 4 groups. They were exposed to infection by inhalation of a strain of Type B influenza virus in the form of a concentrate of virus from allantoic fluid. Clinically recognizable disease, associated with fever of 100° or more, was observed in 41 per cent of the controls, 7 per cent of the group vaccinated 4½ months before infection, 13 per cent of the group vaccinated 4 weeks before, and 11 per cent of those receiving two inoculations, 4½ months and 4 weeks before infection.

The relationship between antibody level and resistance was analyzed and discussed. While 35

per cent of 37 individuals with pre-infection antibody titers of 128 or less developed fever of 100° or more, 9 per cent of 58 subjects having antibody titers of 256 or above had distinct clinical reactions. Moreover, 9 of the former and only 1 of the latter had temperatures exceeding 100.2°.

The restricted antibody response to revaccination within an interval of 4 months, reported in the preceding studies of experimental influenza A, was again noted.

The duration of the immunizing effect of the vaccine appeared to have persisted for at least 4½ months.

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MATURATION AND DESTRUCTION OF TRANSFUSED HUMAN RETICULOCYTES. EVALUATION OF RETICULOCYTE EXPERIMENTS FOR THE MEASUREMENT OF HEMOGLOBIN METABOLISM

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A case of atypical hemolytic anemia, recently investigated in the Strong Memorial Hospital, furnished an unusual opportunity for determination of the *in vivo* maturation time and the fate of transfused human reticulocytes. The results of this study aided in evaluating the usefulness of reticulocyte experiments in quantitative measurements of hemoglobin metabolism.

PREVIOUS QUANTITATIVE ESTIMATES OF THE RATE OF PRODUCTION AND DESTRUCTION OF ERYTHROCYTES

Pigment determinations

In normal animals, there is a fine balance between red cell formation and red cell destruction. Each day as the oldest cells are broken down, their place is taken by new cells which are constantly being released from the bone marrow. Therefore, it ought to be possible to estimate the pace of erythropoiesis by determining the rate of destruction of erythrocytes. This has been admirably accomplished in bile fistula dogs by measurements of the discarded pigment radicles¹ which are excreted quantitatively in the bile (1, 2). The average life of red cells in the dog, estimated in this way (1), is approximately 124 days, which means that less than 1 per cent of the erythrocytes are destroyed and replaced daily.

Transfusion studies

This figure corresponds very well with estimates of the longevity of human red cells as determined by transfusion experiments, in which cells of one type are given to recipients of another (but compatible) type. Earlier transfusion studies, carried out with the original Ashby technique (3 to 5), yielded estimates ranging from 30 to

113 days. However, more recent investigations (6 to 12), employing improved technique and more carefully selected recipients, have resulted in agreement that the youngest of the transfused cells survive for periods of 100 to 140 days, provided the recipient's "hemolytic mechanism" is not abnormal (12).

Reticulocyte experiments

Attempts to measure the daily rate of red cell regeneration and the longevity of erythrocytes have also been made by attacking the problem at the opposite end of the erythroid series; i.e., by following the appearance and maturation of the youngest non-nucleated red cells, the reticulocytes, instead of observing the breakdown of the oldest cells as is done in the pigment and transfusion experiments. On the basis of observations of reticulocytes in rabbits, the average life of red cells has been placed at 8 days (13, 14), at 15 days (15), and at 42 days (16). Others (17) have found that most of the reticulocytes from human cases of anemia become mature within 48 hours. However, these authors estimate that normal human reticulocytes mature within 24 hours and that their life span is 125 days. Baar and Lloyd (18) studied the *in vitro* maturation time of reticulocytes from human cases of hemolytic anemia and from guinea pigs and rabbits with phenylhydrazine anemia. These authors estimate that the average maturation time of reticulocytes is 7 hours but add that it may be longer in cases with reticulocytosis. They (18) utilize the equations of Heilmeyer and Westhäuser (17) and make the following calculations which are based on the assumptions that all red cells enter the circulation from the bone marrow as reticulocytes, that they are not destroyed until they become mature, and that there are approximately

¹ The destruction of 1 gram of hemoglobin is followed by the excretion of approximately 35 mgm. of bilirubin.

0.7 per cent reticulated cells in the peripheral blood of normal human beings:

(1) Percentage of total red cells destroyed and replaced in 24 hours = $(0.7 \times 24)/7 = 2.4$ per cent.

(2) Life span of red cells = $100/2.4 = 42$ days.

These investigators also contend that the above formulae can be applied to the calculation of the average life span of the red cells of any individual, regardless of whether the cells die of senescence or are destroyed indiscriminately without respect to age. An active case of acholuric jaundice is cited as one of a number of examples. This patient had a reticulocyte count of 14 per cent, and the life span of his cells is therefore estimated at 2 to 3 days and the "turnover" of the total red cell mass at nearly 50 per cent per day (19).

At the time the observations of Baar and Lloyd appeared, a very unusual case of atypical hemolytic anemia was being investigated in the hematological laboratories of the Strong Memorial Hospital. Month after month, the percentage of reticulated cells in this patient's circulation remained between 45 and 73 per cent. The formulae of Baar and Lloyd were applied to this case and, to be conservative, a reticulocyte maturation time of 14 hours—twice the average reported by these authors—was assumed. It was then calculated that this patient was destroying and replacing 77 to 120 per cent of her total red cell mass daily, and that the life span of the erythrocytes was only 20 to 30 hours! These figures seemed totally unreasonable. They were even more unacceptable when it was found that the patient's daily output of urobilinogen in the feces was approximately 600 mgm.,² which is only 3 to 10 times the amount excreted by normal persons and is a modest figure for an active case of hemolytic anemia (20).

Disputed maturation time of reticulocytes

Equally disturbing were the conflicting reports of Pepper (21), Heath and Daland (22), Minot and co-workers (23 to 25), and Riddle (26) on the one hand, and Mermod and Dock (27) on the other. Pepper found that reticulocytes could be

demonstrated in rabbit or human blood as long as 48 hours after the beginning of incubation at 37° C. Heath and Daland incubated sterile defibrinated blood from rabbits with phenylhydrazine anemia and from human patients with pernicious anemia at the height of their reticulocyte responses; the human blood was also placed in the pleural cavity of rabbits. When the percentages of reticulated cells were plotted as ordinates and time as abscissa, the results took the form of exponential curves indicating that reticulocytes matured over a period of 4 to 5 days. The curves were the same regardless of the source of the blood and were similar to those later obtained by Heilmeyer and Westhäuser (17).

The work of Minot's group indicates that, in the response to specific treatment of pernicious anemia, the reticulocytes mature within 5 to 10 days and that the magnitude of the reticulocyte response depends largely upon the severity of the anemia. Riddle found that during response to treatment of pernicious anemia the non-reticulated red cells increased in a manner similar to the total red cell count, but lagged behind by at least 2 days. The maturation time of the reticulocytes is therefore estimated at 2 to 4 days.

Mermod and Dock, however, could demonstrate no evidence of maturation of reticulocytes in defibrinated rat and human blood incubated at 37° C. for periods of 24 to 72 hours. Furthermore, these authors state that maturation of reticulocytes in the circulating blood is not proved and seems not to occur on any significant scale. Mermod and Dock also found that reticulocytes are readily destroyed *in vivo* and *in vitro* by various substances, and they suggest that recovery from anemia occurs *in spite of* premature delivery of fragile, short-lived reticulocytes, not because of it.

Consideration of these conflicting observations and opinions furnished the impetus for carrying out the experiments described in this paper. The purposes of this study were:

1. To measure the *in vivo* maturation time of the reticulocytes of the patient with atypical hemolytic anemia when transfused in large quantity to a small child with aplastic anemia;

2. To compare with the *in vivo* results, *in vitro* maturation time of reticulocytes from the same source;

² Some difficulty was encountered in the collection of feces from this patient, but this difficulty was not sufficiently great to alter the interpretation of the above results.

3. To determine, if possible, the fate of the transfused reticulocytes;

4. To evaluate the usefulness of reticulocyte experiments in the quantitative study of the formation and destruction of red blood cells.

METHODS

Counting of reticulocytes

Two methods were used for the enumeration of reticulocytes.

1. "Dry" method. A filtered alcoholic solution of brilliant cresyl blue dye (0.5 gram dye per 100 ml. of absolute ethyl alcohol) was poured over one side of a clean glass slide which was then tilted on end until dry. A small drop of blood (either capillary or oxalated venous blood) was placed in the center of a glass cover slip which was immediately inverted on the slide. Spreading of the drop between the cover slip and the slide was promoted by very light pressure. The cover glass was sealed at once with vaseline and the preparation incubated at 37° C. for 10 to 20 minutes before being examined.³

2. "Wet" method. Blood was drawn into the stem of a Thoma white cell counting pipette to the 0.5 mark and diluted to the 11 mark with a filtered saline solution of brilliant cresyl blue (0.2 gram dye per 100 ml. of 0.6 per cent solution of sodium chloride in distilled water). After being shaken for 30 seconds, the pipette was incubated 10 to 20 minutes at 37° C. The pipette was then shaken again for 30 seconds, several drops were discarded into a piece of gauze, and a small drop of the stained and diluted blood placed on a clean glass slide. A cover slip was then placed over the drop which spread quickly without any pressure, and the preparation was ready for examination. Sealing with vaseline was found unnecessary. This method offered 3 advantages: (1) intimate mixture of dye and cells in the wet state, (2) sufficiently thin preparation, and (3) absence of rouleaux.

Cells stained by either method were viewed slowly through a small hole in a piece of cardboard inserted in the ocular of the microscope. At stated intervals, 1 or 2 counts of 1000 cells each were made by each of the 2 methods; i.e., 2000 to 4000 cells were counted. There was no significant difference in the counts made by the 2 methods.

Use of Simmel's solution

In vitro studies of the maturation of reticulocytes were carried out by a modification of the method of Baar and Lloyd (18) which consisted of incubating blood in sterile, citrated Simmel's (28) solution (NaCl 8.2, KCl 0.2, MgCl₂ 0.2, CaCl₂ 0.2, NaH₂PO₄ 0.1, NaHCO₃ 0.05 grams per liter of distilled water to which one-fifth part of 3.8

per cent sodium citrate solution was added). One ml. of Simmel's solution was placed in each of a number of small, sterile, corked tubes and 4 drops of blood were added to each tube. Whenever counts were made from one of these the remaining tubes were shaken and replaced in the incubator. Staining with brilliant cresyl blue was done by the same methods used for undiluted blood, with the exception that the blood diluted with Simmel's solution was drawn to approximately the middle of the bulb of the white cell pipette instead of the 0.5 mark on the stem. At each interval, 2 samples were taken from one of the tubes and staining was carried out by both the dry and the wet methods.

Differential agglutination

The survival of transfused erythrocytes was followed by use of the Ashby technique (3) as modified by Dacie and Mollison (10). The only deviation from this method was that of preparing an initial 1 in 101 suspension of the recipient's blood instead of a 1 in 51 suspension. Each mixture of serum and cells was prepared in duplicate and a separate count (5 small squares on the improved Neubauer ruling) of the unagglutinated cells in each tube was made by each of 2 persons. The final count recorded was therefore the average of 4 separate counts of each mixture of serum and cells.⁴ Total red blood cell counts were likewise made in duplicate by 2 persons and the average of the 4 counts recorded.

The extremely potent B serum employed was prepared by vaccination of a B donor with A and B specific substances and titrated with A₁, A₂, A₁B, A₂B, and A₃B cells as described elsewhere (29). When this serum, which was used undiluted, was mixed with a pretransfusion sample of the recipient's A₂ cells, there were approximately 15,000 unagglutinated cells per c.mm. The anti-M serum was supplied by Dr. A. S. Wiener and diluted 1:3 with saline before being mixed with cell suspensions. When this serum was added to a pretransfusion sample of the recipient's blood, there were 10,000 unagglutinated cells per c.mm.

Total red cell counts, differential agglutination studies, hemoglobin (photoelectric), and hematocrit (Wintrobe) determinations were made with venous blood drawn without stasis into a mixture of potassium and ammonium oxalate (30) at 37° C. Counts from these tubes were likewise made at frequent intervals by the methods described, and in this way the *in vitro* and *in vivo* maturations of reticulocytes from the same source were compared.

The average figures for percentage of reticulocytes in the recipient's blood are plotted as ordinate and time as

⁴ Control tubes containing 1:201 dilutions of blood in saline were always prepared along with the mixtures of cell suspension and serum. Counts made from these "saline" tubes were nearly always higher than those made from the Thoma standardized diluting pipette. Therefore, the count of unagglutinated cells was always corrected by multiplying this figure by the count from the Thoma pipette and dividing the product by the count from the control tube of saline.

³ Preliminary tests failed to show conclusively that incubation was necessary; nevertheless, this was done because there was no opportunity for further investigation of the necessity of this procedure.

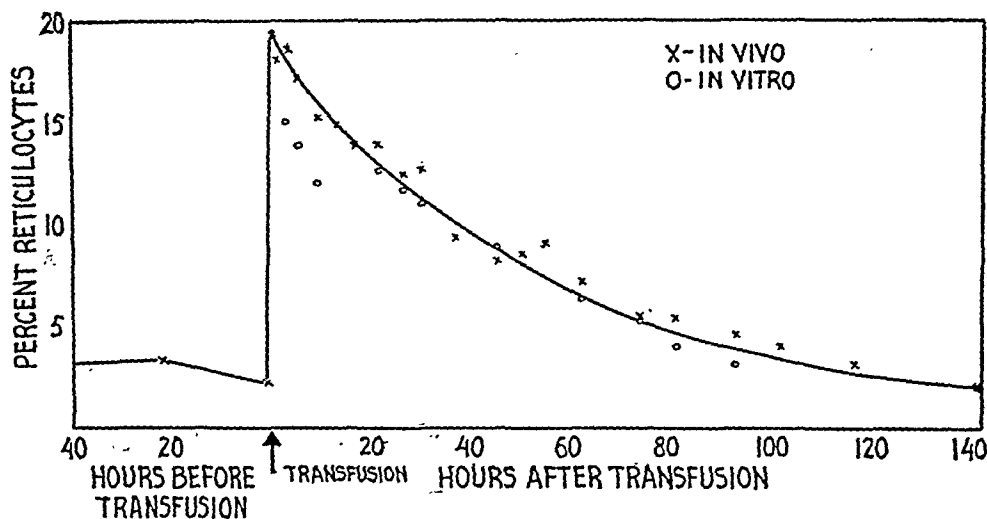


FIG. 1. PERCENTAGE OF RETICULOCYTES IN CIRCULATION OF CHILD WITH APLASTIC ANEMIA BEFORE AND AFTER TRANSFUSION OF RED CELLS FROM ADULT PATIENT WITH HEMOLYTIC ANEMIA

The *in vivo* maturation of reticulocytes is compared with the *in vitro* maturation of cells from the same source when incubated in Simmel's solution.

abscissa in Figure 1. An exponential curve is readily drawn through these points, and it is apparent that the *in vivo* and *in vitro* results are almost identical except for appreciably lower *in vitro* counts during the first few hours of the experiment. Hemolysis of the cells in Simmel's solution was not detectable until after the fourth day. The last reliable *in vitro* counts were made 93 hours after the beginning of incubation of the tubes. During the period of observation, there was not only a gradual decrease in the number of reticulocytes but also a diminution in the amount of vitally staining substance present in the remaining reticulocytes. Moreover, in the incubated cells, there was some degree of replacement of filaments by granules, similar to those described in the *in vitro* studies of others (22, 11, 18).

In vivo destruction of transfused reticulocytes following their maturation

The blood group of the patient with atypical hemolytic anemia was OMNRh +; that of the child with aplastic anemia was A₂MNRh +. When B serum was added to suspensions of the child's red cells after transfusion, the A cells were agglutinated, while the donated O cells were left unagglutinated and could be counted with accuracy by the method described. In this way, it was possible to determine quantitatively the rate at which the transfused cells, most of which were reticulocytes, disappeared from the recipient's circulation.

Calculation of the values for total donated cells, donated reticulocytes, and donated mature (non-reticulated) cells at each interval can be summarized as follows:

- I. Total number donated cells = number cells not agglutinated by B serum minus 15,000.

- II. Number donated reticulocytes = total number donated cells X (total percentage reticulocytes minus 2.5 per cent).

- III. Number donated mature cells = total number donated cells minus number donated reticulocytes.

In formula I, 15,000 cells per c.mm. are subtracted because this was the count of unagglutinated cells prior to transfusion. Formula II is based on the assumption that approximately 2.5 per cent of the reticulocytes present at any given time were the recipient's own cells. All donated red cells are arbitrarily divided into reticulated (immature) and non-reticulated (mature) forms in formula III.

It can be seen in Figure 2 that the total number of donated cells remained nearly constant during the first 2 days after transfusion, and that during this period, there was a marked decrease in reticulocytes and a corresponding increase in mature cells. In the following period of 4 days, there was a rapid fall in the total number of donated cells, and the remaining reticulocytes became mature. The few donated cells remaining at the end of 6 days were free of reticulum and were eliminated during the seventh and eighth days, as demonstrated by further differential agglutination studies.

The comparatively slow destruction of transfused normal cells

Because the length of time the child could remain in the hospital was limited, it was necessary to give her 250 ml. of normal red cells on the fifth and again on the sixth day after the transfusion of reticulocytes. It is clear from Figure 1, however, that the percentage of reticulocytes in the recipient's circulation had returned nearly to the

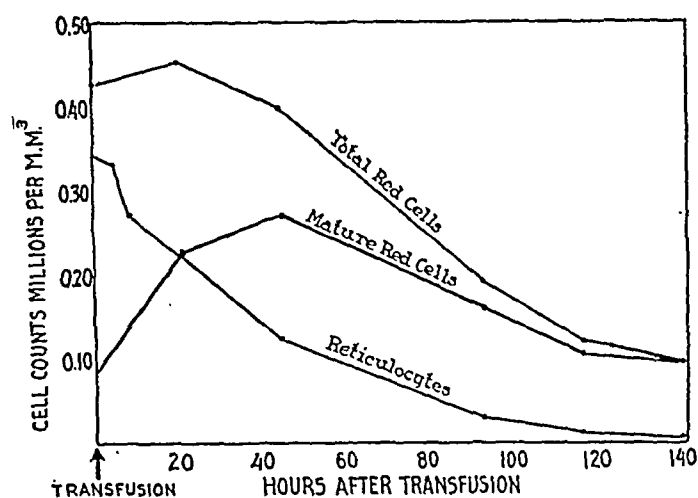


FIG. 2. FATE OF RED CELLS FROM ADULT PATIENT WITH HEMOLYTIC ANEMIA AFTER TRANSFUSION TO CHILD WITH APLASTIC ANEMIA

The few mature cells remaining at 140 hours were destroyed during the next 2 days (Table I). The graph was not extended to show this destruction because large errors are involved in cell counts below 100,000.

original level before the transfusions of normal cells were given. The blood group of the normal cells was ONRh+. It was therefore possible to follow the sur-

vival of these cells by the use of anti-M serum which agglutinated the cells of the recipient (type MN). Calculation of the distribution of cells in the child's circulation was then carried out as follows:

- I. Number ON cells from normal donors = number of cells not agglutinated by anti-M serum minus 10,000.
- II. Number of OMN cells from patient with hemolytic anemia = number of cells not agglutinated by B serum minus 15,000 minus number of ON cells.

The calculated number of OMN cells was less than 20,000 per c.mm. on the seventh day after the transfusion of these cells, and subsequent examinations during the next 37 days confirmed the fact that all of these cells had been eliminated from the child's circulation. During this period, it was shown (Table I) that the count of normal ON cells, which was 2,800,000 on the first day after the last transfusion, had decreased to 2,140,000 in 17 days and to 1,300,000 in 38 days. It was necessary to terminate the study on the thirty-eighth day, and therefore the length of time required for the complete destruction of the normal ON cells cannot be given. However, it appears that, although these cells were destroyed more slowly than the OMN cells, the rate of their elimination was possibly more rapid than that of normal cells in the circulation of recipients with normal hemolytic mecha-

TABLE I
Detailed hematologic observations on blood of child with aplastic anemia before and after transfusion of reticulated OMN cells and normal ON cells

Date	Hour	Reticulo- cytes	Hema- to- crit	Hemo- globin	Red blood cells					
					Total	AMN	Total OMN	Reticu- lated OMN	Mature OMN	ON
		per cent		grams per 100 ml.	millions per c. mm.					
Oct. 3-44	1:30 p.m.	3.1	17.0	6.5	1.85	1.85				
Oct. 4	11:00 a.m.	3.3								
Oct. 5	10:00 a.m.	2.1								
Oct. 5	10:00 a.m.				Transfusion 150 ml. OMN cells, 73 per cent reticulated					
Oct. 5	11:00 a.m.	19.4	22.2	8.4	2.02	1.60	0.42	0.34	0.08	
Oct. 5	12:00 n.	18.8	23.0	8.9	2.09					
Oct. 5	4:00 p.m.	17.0	22.8	8.2	2.29					
Oct. 5	8:00 p.m.	15.7	21.2	7.6	2.06					
Oct. 6	8:00 a.m.	12.9	22.3	7.8	2.15	1.70	0.45	0.22	0.23	
Oct. 7	8:00 a.m.	8.8	21.5	7.9	2.02	1.62	0.40	0.13	0.27	
Oct. 9	8:00 a.m.	4.2	18.0	6.9	1.84	1.65	0.19	0.03	0.16	
Oct. 10	8:00 a.m.	3.3	17.7	6.8	1.72	1.60	0.12	0.01	0.11	
Oct. 10	1:00 p.m.				Transfusion 250 ml. ON cells (Normal)					
Oct. 10	3:00 p.m.		28.3	11.3	2.84	1.22	0.11			1.51
Oct. 11	8:00 a.m.	2.0	28.2	11.3	2.87	1.30	0.10	0	0.10	1.40
Oct. 11	11:30 a.m.				Transfusion 250 ml. ON cells (Normal)					
Oct. 12	8:00 a.m.	0.8	38.0	14.6	4.29	1.47	0.02	0	0.02	2.80
Oct. 13	8:00 a.m.	0.6	38.0	14.6	4.35	1.39	0	0	0	2.96
Oct. 28	9:30 a.m.	1.0	30.7	12.7	3.46	1.32	0	0	0	2.14
Nov. 18	9:30 a.m.	0.9	24.0	10.0	2.58	1.28	0	0	0	1.30

Figures for percentage reticulocytes after transfusion were obtained from curve shown in Figure 1. The relatively small increase in total red cell count produced by the transfusion of OMN cells was due to the fact that the mean corpuscular volume (MCV) of these cells was 138 c. μ . These cells were loosely packed when the 150 ml. volume was measured. The MCV of the ON cells was presumably normal.

nisms (6 to 12). It is therefore possible that there was a small hemolytic element in the anemia of the child whose disease had been labelled "aplastic anemia." Unfortunately, quantitative determination of fecal urobilinogen could not be obtained in order to settle this issue.

A second determination of the maturation time of reticulocytes in vitro

On October 17, 1944, 20 ml. of blood were drawn from M. G., the patient with atypical hemolytic anemia. A 10 ml. portion was defibrinated by gentle shaking with a small number of glass beads in an Erlenmeyer flask, after which, 1 ml. of the defibrinated blood was placed in each of 8 small, sterile, tightly-stoppered tubes. No grossly detectable hemolysis was produced by this procedure. The remainder of the whole blood was distributed among tubes of Simmel's solution by the method previously described. All but one of the tubes of each type were incubated at 37° C.

At intervals of 7 to 26 hours during the next 6 days, one tube of defibrinated blood and one tube of blood diluted with Simmel's solution were removed from the incubator for examination, and the remaining tubes were shaken and replaced in the incubator. Both "wet" and "dry" preparations were made with brilliant cresyl blue and the reticulocytes counted as before. During the first 48 hours of incubation, the percentage of reticulated cells in the defibrinated blood was not significantly different from that of the cells suspended in Simmel's solution. The average of the counts from both tubes at each interval is therefore recorded in Figure 3. After 48 hours, however, there was considerable hemolysis of the defibrinated blood and counts made from the defibrinated samples after that time were considered unreliable. The remainder of the graph shown in Figure 3 is therefore based only upon the counts of the reticulated cells in Simmel's solution in which there was but slight hemolysis on the fifth and sixth days. In spite of the fact that the reticulocytes were enumerated at relatively long intervals and that the defibrinated blood proved unsatisfactory, this rather crude *in vitro* experiment served to confirm the previously obtained results.

At the time blood was drawn for this experiment, the percentage of reticulocytes in the patient's blood had decreased to 29.6, presumably as a result of the transfusion given 12 days previously. However, the exponential curve showing the maturation of reticulocytes during a period of 6 days is very similar to that obtained as a result of the earlier combined *in vivo* and *in vitro* study.

DISCUSSION

In the case presented in this paper, the child's reticulocyte count was raised by nearly 17 per cent as a result of transfusion, and the gradual decline in the percentage of reticulated cells took place over a period of approximately 140 hours, or roughly 5 to 6 days. Baar and Lloyd (18), who

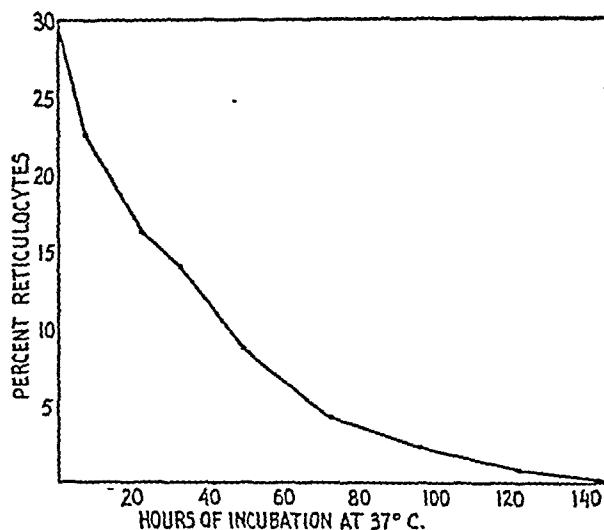


FIG. 3. *IN VITRO* MATURATION OF RETICULOCYTES FROM PATIENT WITH HEMOLYTIC ANEMIA.]

performed the only previous experiment of this type, produced an increase of 5.4 per cent in the reticulocyte count of an infant by transfusing blood from a patient with acholuric jaundice. The *in vitro* maturation time of reticulocytes from this patient is recorded by these authors as 6 hours, which is approximately the time required for the *in vivo* maturation of the transfused reticulocytes in the circulation of the infant.

There are 3 possible explanations for the marked discrepancy in these 2 estimates of maturation time.

1. The experimental error in the counting of reticulocytes is known to be large; this is particularly true when dealing with numbers as small as 5.4 per cent. Heath and Daland (22) admit that no conclusions can be drawn from their own transfusion experiments in which the reticulocyte counts of rabbits were raised by only 3 to 6 per cent.

2. Baar and Lloyd (18) determine maturation time by extrapolation of the initial steep slope of the maturation curve to the ordinate zero. It is difficult to follow the reasons given by these authors for using this procedure which, if applied to the reticulated cells of Figures 1 and 3, might yield estimates as low as 20 to 40 hours. It seems more reasonable to consider the interval between the beginning of the experiment and the hour at which the base-line is first reached, as the maturation time of the youngest reticulocytes.

3. The length of time required for a reticulated cell to lose its vitally staining substance may vary somewhat depending upon its source. Baar and Lloyd suggest that this period may be relatively long in cases with reticulocytosis and short in cases of pernicious anemia. Heilmeyer and Westhäuser (17) found that maturation curves vary somewhat depending upon the source of the blood, but in all cases, the general trend is the same. Riddle (26) believes it likely that in normal and less anemic individuals, the reticulocytes are retained longer in the marrow and developed nearer to maturity before release, and may therefore require less time in the circulation to lose their reticulum.

Comparison of in vivo and in vitro studies of the maturation of reticulocytes

Among those who have compared the *in vivo* and *in vitro* maturation of reticulocytes (18, 22), there is general agreement that the results are essentially the same by either method. Not only are similar estimates of maturation time obtained, but the same sort of exponential maturation curve can be plotted with figures obtained in either way. This can also be said of the data presented in this paper. It should be emphasized, however, that the *in vivo* observations recorded here were obtained by utilizing a case that was far more advantageous than any previously investigated. The correlation of the *in vivo* and *in vitro* results in this case is therefore of special interest.

Fate of transfused reticulocytes

It has already been pointed out that during the first 2 days after the transfusion, the total count of donated cells fell but little, while there was a marked decrease in percentage of reticulocytes (Figure 2). It can therefore be concluded that during this interval many of the reticulated cells became mature. During the first 2 days, there was a preponderance of maturation and a minimum of red cell destruction, but during the next 4 days, after most of the cells had become mature, the reverse was true. These findings are sufficient to disprove the contention of Mermod and Dock (27) that reticulocytes do not mature in the circulating blood. These investigators argue that the reticulum merely becomes more difficult to stain and that these cells do not really mature with in-

cubation. They attribute the results of Heath and Daland (22) to the use of "dry" staining technique. However, in the present study both wet and dry techniques were used with nearly identical results.

The observations shown in Figure 2 support the opinion of Baar and Lloyd (18) that reticulocytes are not destroyed until they become mature and they are also in keeping with Cruz' observation that reticulocytes are not destroyed by acetylphenylhydrazine (32). At present, it is possible only to speculate on the explanation for this peculiar immunity to destruction.

It must also be admitted that under certain conditions reticulocytes may be destroyed as readily, or perhaps more readily, than mature cells. Rhoads *et al.* (33) produced hemolytic anemia with indol in dogs fed deficient diets and attributed the lack of reticulocytosis to rapid destruction of immature cells. Dock (34) found doses of saponin which on injection into rabbits destroyed circulating reticulocytes with negligible effect on mature cells, and Dock and Mermod (35) demonstrated a reticulocyte lysis in the serum of active cases of pernicious anemia. It has been suggested that such a lysis is responsible for increased pigment excretion and lack of reticulocytosis in this disease before specific therapy is given.

Defectiveness of reticulocytes

The red cells of the patient with atypical hemolytic anemia are destroyed rapidly, not only in her own body but after transfusion to a child whose circulation retains normal erythrocytes for much longer periods. These facts indicate that the cells of M. G.⁵ are abnormal, but there is uncertainty as to whether they are structurally defective in the same sense as spherocytes and sickle cells, or defective merely because they happen to be released from the marrow in the reticulated form. It is reasonable to suspect that in certain anemias, the marrow is working under difficulties and therefore turns out cells with defective stroma, as

⁵ Normal ON cells were transfused to patient M. G. on 2 separate occasions. The donated erythrocytes survived 114 days after the first transfusion, and 790,000 donated cells per c.mm. were counted 39 days after the second transfusion (31).

suggested by Whipple (36). There is no proof, however, that such defectiveness is limited to cells which leave the marrow in the reticulated stage. There is also no evidence that under normal conditions cells entering the circulation in this earlier stage of development have any shorter life expectancy than those leaving the marrow without vitally staining substance.

It is virtually impossible to study the behavior of reticulocytes from normal human beings and lower animals because they are present in the blood in very small numbers. All published observations on reticulocytes, including those described in this report, have been made with blood drawn from abnormal mammals or normal mammals subjected to abnormal conditions. *Great caution must therefore be taken in applying these results to the behavior of the normal erythron.*

Use of reticulocyte experiments in the study of hemoglobin metabolism

It is of interest to compute the life span of the red cells of patient M. G. by using the formulae of Heilmeyer and Westhäuser (17) and Baar and Lloyd (18). If the reticulocyte count is 73 per cent^o and the maturation time 140 hours, then the daily rate of regeneration of red cells is $(73 \times 24)/140 = 12.5$ per cent and the average life span of the cells is $100/12.5 = 8$ days.

This figure coincides with the 8-day period of survival actually observed after transfusion of the cells into the child. This correlation indicates that in the case of M. G. nearly all of the cells were leaving the marrow in the reticulated stage and were not destroyed until they had matured. It will be recalled that these were the assumptions on which the above formulae were based.

Prior to the transfusion experiment the body weight of patient M. G. was 67 kgm., estimated blood volume approximately 6000 ml., hemoglobin 9.5 grams per 100 ml., and total circulating hemoglobin 570 grams. If the entire red cell mass was being broken down and replaced every 8 days, then the calculated rate of hemoglobin "turn-over" is 71 grams per day. This figure is approxi-

mately 10 times that (7.2 grams per day) calculated for a normal person of the same body weight but with 15 grams of hemoglobin per 100 ml. of blood and with a red cell longevity of 125 days.

If 35 mgm. of bilirubin are formed from the breakdown of each gram of hemoglobin, the calculated rate of bilirubin excretion from the common bile duct of patient M. G. is 2485 mgm. per day, as compared with 252 mgm. per day in the normal individual of the same body weight. The quantitative elimination of bilirubin in bile fistula dogs has been amply demonstrated (1, 2), but the ratio between output of bilirubin in the bile and excretion of urobilinogen in the feces has never been accurately determined. No doubt this ratio is subject to considerable variation under normal as well as abnormal conditions. According to Watson (20), the fecal urobilinogen excretion of normal adults varies from 40 to 280 mgm. per day (usually 100 to 200 mgm.), while in cases of hemolytic anemia, values ranging from 300 to 4000 mgm. per day (usually 600 to 2000 mgm.) are recorded.

In June 1944, when the reticulocyte count was 62 per cent, urobilinogen was excreted in the feces of M. G. at the rate of 600 mgm. per day. However, the fecal collections were rather unsatisfactory and this figure is probably much below the true value. The need for accurate determinations of fecal urobilinogen excretion in conjunction with transfusion experiments of the type reported in this paper is apparent.

If the maturation time of all reticulocytes is approximately 140 hours, as previously suggested, and the normal average reticulocyte count in human beings is 0.7 per cent, then the normal average daily rate of red cell regeneration is $(0.7 \times 24)/140 = 0.12$ per cent and the average life span of the cells is $100/0.12 = 833$ days.

This estimate is 7 to 8 times greater than those based on pigment studies and transfusion experiments, the results of which are not acceptable to Baar and Lloyd for reasons that are difficult to follow (19, 37). These authors, however, place the average life span of normal erythrocytes at 42 days which is nearly 20 times lower than the figure of 833 days computed with their formulae.

There are 3 possible explanations for the failure of these calculations to provide a reasonable estimate of the longevity of red cells:

^o The percentage of M. G.'s cells that are reticulated is 80, if calculated from the data shown graphically in Figure 2. However, this figure is obviously less accurate than the direct count of 73 per cent made just before the blood was transfused.

1. Determinations of the maturation time of reticulocytes may be erroneous. The 140 hour figure has already been defended and nothing more can be added here.

2. Reticulocytes may be destroyed before they become mature. However, it is unlikely that this takes place in normal individuals and it is reasonably certain that this did not occur in the case which formed the basis for this report.

3. By far the most important reason for the incongruity of the figures calculated according to the equation of Baar and Lloyd, and for the failure of reticulocyte experiments to measure the tempo of hemoglobin metabolism, is that *not all red cells enter the circulation in the reticulated form*.

This belief is supported by the classic observations of Minot's group who conclude that only in the more anemic patients with pernicious anemia can the initial increase in total red count be attributed entirely to the production of reticulocytes following specific therapy. These investigators add that since the total red cell count continues to rise after the percentage of reticulocytes has reached a peak and returned again to a normal level, it must be concluded that enough time has elapsed so that most of the erythrocytes are mature when released from the marrow. Moreover, in less anemic patients, even the initial increase in total red cells is due largely to the release of erythrocytes which are already mature when they enter the circulation.

Additional evidence is furnished by the observations of Rioch and Robscheit-Robbins (38) who found no correlation between reticulocyte formation and output of hemoglobin in standard anemic dogs.

In view of these facts, it is likely that, under normal conditions, only a small percentage of the red cells delivered from the marrow contains detectable amounts of vitally staining substance. This is the chief reason for the distinctly limited usefulness of reticulocyte experiments in estimating the daily rate of red cell regeneration and the life span of erythrocytes.

SUMMARY

1. Approximately 150 ml. of red cells, 73 per cent of which were reticulated, were removed from a patient with atypical hemolytic anemia and

transfused to a child with probable aplastic anemia. The number of reticulocytes in the child's circulation was thus raised by 17 per cent.

2. The transfused reticulocytes gradually matured over a period of about 140 hours. A similar maturation curve and estimate of maturation time were obtained by *in vitro* incubation of the same blood in Simmel's solution.

3. All of the transfused cells were destroyed in approximately 8 days, but it was demonstrated that the reticulocytes were not destroyed until they had become mature. It is emphasized that these cells were abnormal and that for this reason the results of this experiment must be applied with great caution to the behavior of the normal erythron.

4. It is concluded that reticulocyte experiments have only limited usefulness in estimating the daily rate of red cell regeneration and the life span of erythrocytes because:

- a. The maturation time and fate of reticulocytes may vary, depending upon the conditions under which they are produced;
- b. Under certain circumstances, reticulocytes may be destroyed before they become mature;
- c. Under normal conditions and in some phases of the response to anemia, most of the red cells probably enter the circulation in the non-reticulated form.

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CALCIUM METABOLISM IN NEPHROSIS. I. A DESCRIPTION OF AN ABNORMALITY IN CALCIUM METABOLISM IN CHILDREN WITH NEPHROSIS

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In 1923, not many years after Epstein's (1) original characterization of lipid nephrosis, Salvesen and Linder (2) first observed a lowering of the serum calcium concentration which paralleled the decrease in serum protein level in patients with this disease.¹ In addition, two other features indicating a disturbance of calcium metabolism in nephrosis have been described. Boyd, Courtney, and MacLachan in 1926 (5) and Scriver in 1928 (6) both noted an excessive loss of calcium in the feces and a diminished excretion in the urine.

In the course of a systematic study of children with nephrosis in this hospital over the past 5 years, a further indication of abnormal calcium metabolism has come to our attention, namely, a generalized decalcification of the skeleton as observed by x-ray. The degree of this decalcification varies roughly with the severity and duration of the disease; in 2 instances in our experience, fractures have occurred after relatively slight trauma. Some degree of decalcification is often demonstrable within a month after detection of the disease.

The studies reported in this and a subsequent paper were designed to correlate the x-ray changes

in the bones with the alterations in calcium metabolism.

SUBJECTS

Two boys, S. G. aged 11 years, and R. M. aged 4 years, and one girl, R. Q. aged 9 years, all exhibiting the signs and symptoms of the nephrotic syndrome or lipid nephrosis were selected for study. Protocols of the subjects are shown graphically in Figure 1. Each patient had been observed in this hospital for at least 2 years prior to the period of this investigation, which was carried out during the last 6 months shown in the graphic protocols.

Subject S. G., a few months prior to this study, had begun to exhibit diminution of both the urea clearance test and the ability to concentrate the urine. In subjects R. M. and R. Q., these functions were normal. Throughout the 6-month period of study, the condition of R. Q. and S. G. remained stationary, but in the second month, R. M. underwent a spontaneous remission from his disease following a pneumococcus Type IV bacteremia. It was possible to maintain the constant metabolic regimen throughout the recovery period. During his subsequent convalescence, he was occasionally utilized as a control, designated as "convalescent," showing a normal calcium metabolism and at the same time a degree of skeletal calcium deficiency comparable to that of the other patients. Another boy, J. P., aged 5 years, who had recovered from an attack of acute glomerulonephritis 2 months previously, was also used as a control, designated "normal" (see Figure 1).

METHOD OF STUDY

Each subject was placed on a high-protein, low-sodium diet of constant weekly composition, adequate in calories and having a slightly alkaline ash. Vitamins were supplied by 15 drops of percomorph oil, 100 ml. of orange juice, and 10 grams of brewers' yeast daily. The dietary calcium, 75 per cent of which was in the form of milk,

¹ These authors, accepting the theory that part of the serum calcium is bound to the proteins and thereby rendered non-ionizable and non-diffusible, came to the conclusion that the deficiency in the serum calcium was entirely in this fraction, and that the ionized or diffusible portion was at a normal concentration. They based their conclusion on the facts that tetany never occurred in these patients, even when the total serum calcium level fell to "tetanic" levels, and that edema fluid contained as high a concentration of calcium in nephrotic patients as in cardiac patients who had a normal serum calcium level. Since then, Liu (3) has shown by ultrafiltration that the diffusible calcium in the serum of these subjects is within normal limits, and McLean and Hastings (4) with the frog-heart technique have demonstrated that the ionized calcium is also normal.

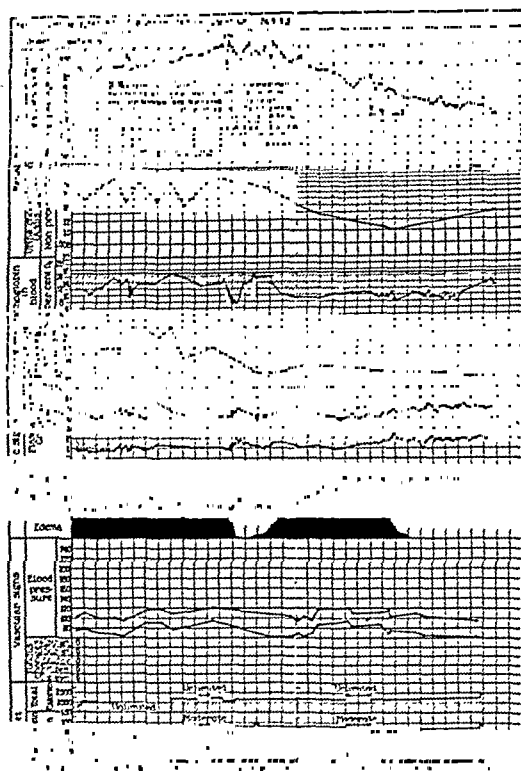


FIG. 1A

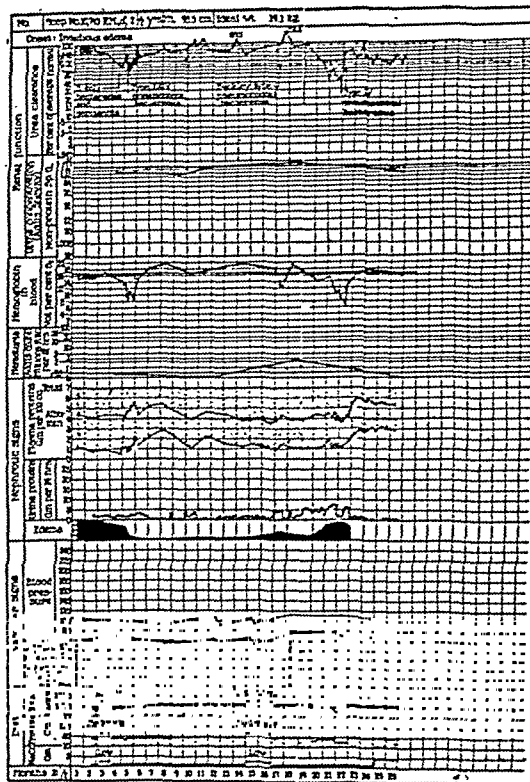


FIG. 1C

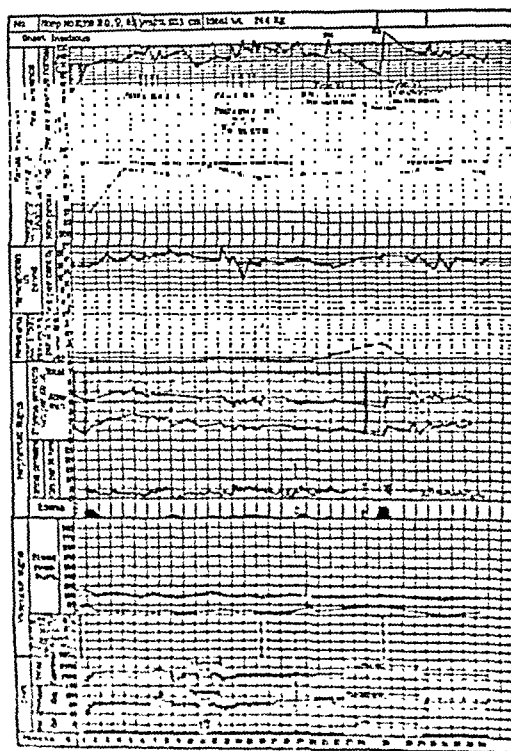


FIG. 1B

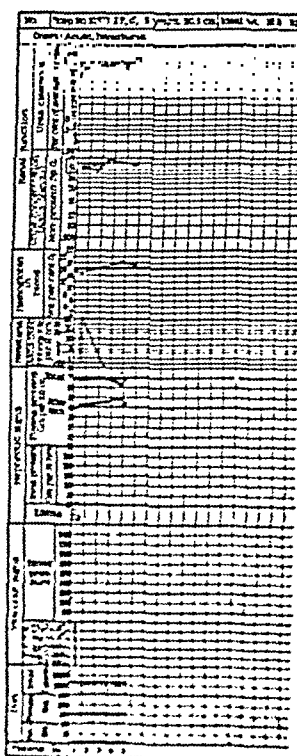


FIG. 1D

FIG. 1. DATA SUMMARIZING LABORATORY AND CLINICAL OBSERVATIONS ON S. G. R. Q. AND R. M. THROUGHOUT THE OBSERVED COURSE OF THEIR ILLNESS

The present studies were carried out during the last 6 months shown in the figures. The fourth protocol is that of the normal control (J. P.).

varied between 0.7 and 1 gram, and the phosphorus between 0.9 and 1.3 grams daily. After preliminary control studies, patient S. G. received 1 gram of added calcium in his diet as calcium lactate.

COLLECTION OF SPECIMENS

One-half portions of each diet as served were weighed, mixed in a Waring blender, transferred to a large evaporating dish and evaporated to dryness on a steam bath to a constant weight. The dry material was pulverized with a mortar and pestle. Duplicate 1-gram samples were weighed out for nitrogen analysis. The nitrogen content of the diets prepared for analysis in this manner never varied more than 1 per cent from the value calculated from standard tables of food analysis. Four-gram samples were used for dry ashing, which was carried out overnight in a muffle furnace at 500° C. The ash was dissolved in hot normal HCl, transferred quantitatively to a 100 ml. volumetric flask, cooled, made up to volume, and filtered through ash-free filter paper. Analyses for Ca, P, Na, K, and Mg were performed on the filtrate.

The stools were marked off with 0.4 gram of carmine given at the beginning of each experimental period. Each stool was transferred quantitatively from the container into which it was passed to a Waring blender. After thorough blending, the stool was again transferred quantitatively to a 5 liter bottle, previously weighed to 0.1 gram. At the end of the collection period, the bottle was again weighed to within 0.1 gram, the difference between the two weights being the wet weight of the stool plus the water used in transfer. It was then shaken for 15 minutes on a shaking machine and 10 ml. samples measured in quadruplicate into weighing bottles from a calibrated wide-mouthed pipette, allowing 5 minutes for the pipette to drain. These samples were weighed and then dried on a steam bath to a constant weight. The total dry weight of the stool was then calculated by multiplying the total wet weight by the dry weight/wet weight ratio.

The remainder of the stool was dried on a steam bath and pulverized with a mortar and pestle. The total dry weight for each metabolic period was calculated from the total wet weight \times the dry weight/wet weight ratio. Duplicate 2-gram samples were ashed and dissolved in 100 ml. of normal HCl using the technique described above for the diets. Mineral analysis of the duplicate ash solution agreed within 1 per cent.

The urine for each metabolic period was pooled, the volume measured, and chloride and nitrogen measurements made. For other analyses, 400 ml. of urine were brought to dryness on a steam bath, ashed, and redissolved in 100 ml. normal HCl with the technique used for diet and stools.

ANALYTICAL METHODS

Calcium was determined on 10 ml. of urine ash (40 ml. of urine) and 2 ml. of stool ash solution according to the procedure of Tisdall and Kramer (7). Because of the extremely low urinary calcium excretion, some samples

were checked by isolating and analyzing all the calcium in 200 ml. urine samples. Similar values were obtained in both cases. Serum calcium was measured by the manometric method of Van Slyke and Sendroy (8). Magnesium was determined on 3 ml. samples of the calcium-free supernatants by the hydroxyquinolate method as outlined by Nordbö (9). Sodium was determined by the method of Butler and Tutthill (10). Only a few stool sodiums were done because the values were so low as to be negligible. Nitrogen was determined in stools and urine by the macro-Kjeldahl procedure (11). Plasma albumin and globulin were measured by the micro-Kjeldahl method (11). Phosphorus was measured by the Fiske and Subbarow (12) method, and potassium by the method of Fiske and Litaczek (13).

For the determination of fecal lipids, two 1-gram samples of dried stool were placed in 200 ml. volumetric flasks; one sample was used for estimation of neutral fat and the other was acidified with 1 ml. of concentrated HCl for the analysis of total fecal lipids. The volume of each sample was increased to 200 ml. with equal parts of redistilled absolute alcohol and ether and extraction allowed to proceed for 2 hours at room temperature with occasional gentle shaking. After filtering, an aliquot of the filtrate was evaporated to dryness on a steam bath and analyzed for carbon according to the technique of Van Slyke and Folch (14) by wet combustion and manometric measurement of the CO₂ produced.

OBSERVATIONS

I. X-ray

The nature of the bone changes seen in nephrosis is brought out by the contrast between the roentgenogram of the hand of a child with nephrosis and that of a normal child of similar age and development which were photographed simultaneously on the same plate (Figure 2). In the patient (No. 2), the carpals and the shafts of the phalanges, metacarpals, radius, and ulna exhibit a diffuse rarefaction, notwithstanding the fact that the epiphyseal lines are as dense as those of the normal subject. This picture suggests that, although epiphyseal growth and calcification proceed normally, for some reason there is an excessive loss of calcium from the shafts of the bones. In other words, in contrast to rickets, the difficulty in nephrosis appears not to be an inability to deposit calcium in the osteoid matrix formed at the epiphysis, but rather an inability to hold the calcium in the shafts of the bones despite adequate calcification of the epiphyseal matrix. The majority of the children with nephrosis studied in this clinic have grown at a normal rate during their illness and not one has shown any



FIG. 2. X-RAY COMPARISON OF THE HAND OF A NEPHROTIC CHILD (S. G., No. 2) WITH THE HAND OF A NORMAL CHILD (No. 1) OF SIMILAR AGE AND DEVELOPMENT, PHOTOGRAPHED SIMULTANEOUSLY ON THE SAME PLATE

sign of rickets or other skeletal disease. Even when fractures occurred, healing proceeded normally.

II. Metabolic studies

The metabolic data obtained on the 3 patients, which are summarized in Table I along with those of the normal control, confirm the findings of previous workers (2, 5, 6). The virtual absence of calcium from the urine is perhaps the most striking observation. We have analyzed 24-hour urine specimens of 6 other patients with this disease, not included in this report, and in all obtained urinary calcium values of similar small magnitude.

Our patients showed the characteristic low serum calcium concentration common to the disease. With the exception of S. G., who had signs of renal functional insufficiency, usually accompanied by phosphorus retention, the concentration of phosphorus in the serum was normal for children of this age. It should also be mentioned that all these patients had normal levels of serum phosphatase.²

The fecal calcium was only a little less than the calcium intake, resulting in extremely low calcium retentions. It is worthy of note that in none of

² We are grateful to Dr. Lester M. Smith, M.D., of the General Hospital, Boston, Massachusetts, for these measurements.

TABLE 1

Average daily nitrogen, calcium, and phosphorus balances in children with nephrosis

Patient and date	Nitrogen				Calcium				Phosphorus				Serum		Total plasma protein
	Intake	Feces	Urine	Balance	Intake	Feces	Urine	Balance	Intake	Feces	Urine	Balance	Calcium	Phosphorus	
	grams per day				grams per day				grams per day				mgm. per 100 ml.		
J. G. ♂ 11 yrs. Oct. 11–Oct. 30	12.620	1.140	10.130	+1.350	0.736	0.728	0	+0.008	1.195	0.794	0.420	+0.283	6.60	7.30	4.84
L. Q. ♀ 9 yrs. Feb. 2–17	12.780	0.866	10.330	+1.584	0.993	0.875	0.006	+0.112	1.444	0.520	0.623	+0.299	8.74	5.60	4.56
R. M. ♂ 4 yrs. Dec. 30–Jan. 13 before crisis)	6.680	0.925	5.800	−0.015	0.823	0.807	0.001	+0.015	0.852	0.630	0.290	+0.054	7.09	4.99	4.34
Feb. 24–March 10 after crisis)	7.460	0.530	5.760	+1.170	0.917	0.432	0.032	+0.453	0.928	0.290	0.455	+0.183	9.20	4.60	5.90
J. P. ♂ 5 yrs. May 12–19 normal control)	9.240	0.792	7.280	+1.168	1.077	0.717	0.092	+0.268	1.119	0.320	0.670	+0.129	10.5	5.00	6.51

† Average of weekly analyses.

our patients was the excessive fecal calcium excretion associated with steatorrhea. The amount of fat in the stools was within the normal range and of the same magnitude as that in the control subject.

The fact that disturbances in nitrogen and mineral metabolism are a definite part of the nephrotic syndrome is well illustrated by the data obtained on patient R. M. before and after a "nephrotic crisis" (15) associated with a Type IV pneumococcus bacteriemia which was followed by a remission from his disease. The metabolic events which occurred at the time of the "crisis" are described by the data shown graphically in Figure 3, and they provide a striking account of a remission in nephrosis. Prior to the "crisis," the patient was extremely edematous. He had the characteristic hypoproteinemia and hypocalcemia of nephrosis. The urinary excretion of sodium averaged less than 2 m.eq. and of calcium no more than 1 mgm. daily. On the other hand, the fecal loss of nitrogen, calcium, and phosphorus was of such a magnitude that the child was in a negative balance in respect to nitrogen and phosphorus, and retained only 2 per cent of his ingested calcium. The high fecal phosphorus excretion resulted in a reversal of the normal fecal:urinary phosphorus ratio of 2:3 (16).

Following the "crisis" there was a diuresis, with a loss of 6.5 kgm. in weight and complete disappearance of edema. At the same time, the

serum protein and calcium levels rose simultaneously to near normal values. The diuresis was accompanied by a remarkable outpouring of sodium, calcium, and phosphorus in the urine. The average daily urinary sodium excretion during the

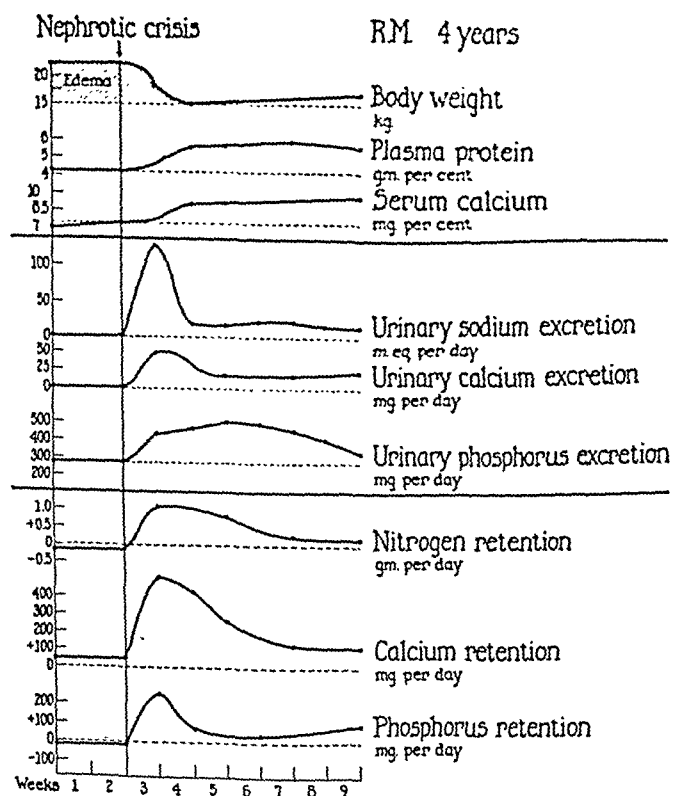


FIG. 3. A GRAPHIC REPRESENTATION OF THE METABOLIC EVENTS OCCURRING AT THE TIME OF A NEPHROTIC "CRISIS" ASSOCIATED WITH A TYPE IV PNEUMOCOCCAL BACTERIEMIA IN PATIENT R. M.

2 weeks after the "crisis" was 60 m.eq. greater than the intake, and the average daily weight loss 450 grams. Since 450 grams of interstitial fluid contain 65 m.eq. of sodium, it is apparent that this patient's weight loss could be almost entirely accounted for by a loss of interstitial fluid.

Owing to the sharp decrease in fecal excretion, there was also a tremendous increase in total calcium retention, notwithstanding the marked increase in the urinary output of this element. The total retention of nitrogen and phosphorus was likewise greatly increased, and the fecal:urinary phosphorus ratio returned to normal (Table I). In this respect, the patient behaved like a child who, after a prolonged period of starvation, was again receiving an adequate diet. The rapid shift of calcium and phosphorus excretion from stool to urine, which would not be expected to occur during recovery from starvation, is probably a secondary result of the correction of the abnormal water retention in nephrosis. Apparently, the calcium and phosphorus contained in the edema fluid were in large measure washed out along with the sodium during the diuresis. After the edema had subsided, the urinary calcium excretion fell to low normal values which would be expected in the presence of a skeletal calcium deficiency.

The combination of low serum calcium concentration, extremely low urinary calcium excretion, and abnormally low calcium retention has been encountered in all the nephrotic children that have been studied, both by others (5) and by us. This combination appears to be characteristic of the disease. In case R. M. (Table I and Figure 3), all these abnormalities of calcium metabolism present before the "crisis" entirely disappeared after the "crisis," together with the clinical signs of nephrosis other than albuminuria. This occurrence adds to the evidence that the calcium abnormalities are a part of the nephrotic syndrome.

DISCUSSION

Three facts appear from the observations presented: I. The shafts of the bones showed rarefaction by x-ray examination, while, on the contrary, the density of the epiphyseal lines was relatively normal. II. Skeletal growth was normal, in so far as bone length and shape were concerned. III. The nephrotic children failed to retain calcium at a rate even remotely approaching the re-

tention rates of normal children. In 2 of the 3 cases, fecal excretion of calcium almost exactly equaled calcium intake. Almost no calcium was excreted in the urine. Apparently, the renal retention was compensatory; without it, the calcium deficit of the organism would have been more severe.

The actual rates of calcium storage per kgm. body weight for all our experimental subjects, including R. M. before and after his "crisis," have been calculated from the data in Table I and are presented in Table II. Additional data obtained in the case of S. G. after his dietary calcium had been more than doubled are also given in Table II. To compare graphically the annual rate of calcium storage of these children on their standard diets throughout the course of their illness with the annual rate of a normally growing child, as given by Leitch (17), Figure 4 has been constructed by assuming that the total annual increase in body calcium equals the average daily calcium retention per kgm. ideal body weight \times average annual body weight \times 365. The data for calculating the annual rate of calcium storage in the children with nephrosis were taken from Table II. Patient S. G. presents an extreme example of the total calcium deficit which can occur in these children over the course of time. His annual calcium storage rate shown in the graph between the ages of 9 and 11 years was calculated from the data obtained when his intake was 0.736 gram of calcium per day which is approximately the amount of calcium provided by the high-protein, low-salt diet employed in the treatment of nephrosis in this hospital. On this intake, S. G. retained 8 mgm. of calcium daily. At that rate, he would store only 2.85 grams of calcium in a year. When his intake was increased to 2.189 grams of calcium per day, the calcium retention rose to 220 mgm. per day (Table II), or 80 grams per year, as shown in the graph for the period between 11 and 12 years. Leitch found that an average of 120 grams was stored annually by a child of the same age.²

² Leitch's figures agree with those of Sherman (18) but are higher than others in the literature, namely those of Kinsman *et al.* (19), Dackworth (20), Sells (21), and Macy (22). R. Q. and R. M. however, show deficient calcium storage by any published standard and only S. G. on the high intake of 2 grams daily reaches

TABLE II

Average daily calcium retention per kilogram ideal body weight in 3 children with nephrosis (including one convalescent after a "nephrotic crisis") and one normal child

Patient	Date	Actual weight	Ideal weight for height	Calcium			
				Intake	Balance	Retention	
						Per kgm. ideal weight	Percentage of intake
		kgm.	kgm.	grams per day	grams per day	grams per day	per cent
S. G. 11 yrs.	Oct. 16-Oct. 30	26.7	26.3	0.736	+0.008	0.0003	1
	Dec. 30-Jan. 13 ¹	25.8	26.3	2.180	+0.220	0.008	10
R. Q. 9 yrs.	Feb. 3-Feb. 17	27.0	30.4	0.993	+0.112	0.001	11
R. M. 4 yrs. (Before "crisis")	Dec. 30-Jan. 13	22.0	16.4	0.823	+0.015	0.001	2
	1 week after "crisis"	15.5	16.4	0.948	+0.481	0.030	51
	4 weeks after "crisis"	15.2	16.4	0.948	+0.215	0.013	22
J. P. 5 yrs. Normal	May 12-May 19	20.2	18.6	1.077	+0.268	0.014	25

¹ 7.5 grams of calcium lactate per day added to diet.

In spite of their deficient calcium retention, 2 of the nephrotic subjects, R. Q. and R. M., grew at a perfectly normal rate, as measured by the

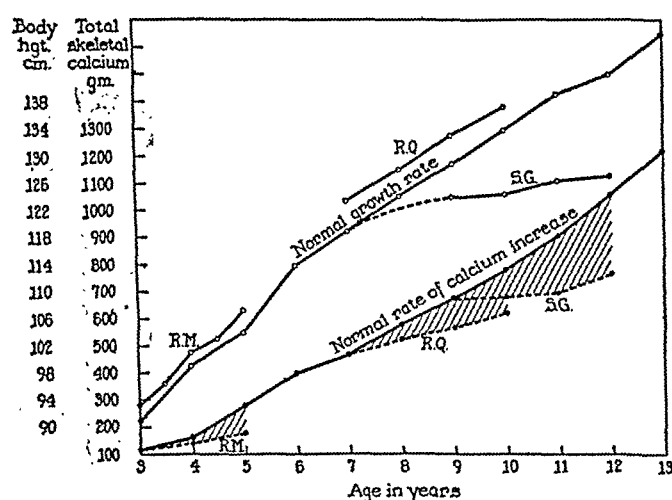


FIG. 4. RATE OF GROWTH AND CALCIUM STORAGE IN NORMAL CHILDREN AND IN 3 CHILDREN WITH NEPHROSIS, S. G.,* R. Q., AND R. M., THROUGHOUT THE COURSE OF THEIR ILLNESS

Normal values taken from Leitch (17). Values for patients calculated from data in Table II.

* S. G.'s calcium storage rate during his 9th and 10th years was calculated from the data obtained while he was on a daily intake of 0.736 gram of calcium; during his 11th year the data obtained while he was on a daily intake of 2.197 grams were used (see Table II).

the lower storage levels observed in normal children on half that intake.

Baldwin-Wood standards for height (23), during the entire period of their illness. S. G., the child who failed to grow normally, was the one whose urea clearance test and urine concentrating ability were diminished.⁴ All 3 children presented the same degree of skeletal decalcification. These facts are important in indicating that the decalcification is not the result of a generalized nutritional disturbance with failure to form new bone matrix, but rather an inability to retain calcium in the shafts even though it was deposited at the epiphyseal lines.

Of the foregoing facts, the following may be related in a direct sequence of cause and effect: (1) the excessive fecal loss of calcium, (2) the failure to retain calcium at a normal rate, and (3) the rarefaction of the shafts of the long bones. Two additional facts are of importance in describ-

⁴ In the 2 years since the completion of this study, patient S. G. has taken a sudden spurt in growth of 8 cm. in contrast to a growth of only 4 cm. in the 3 preceding years. At the same time, his x-rays indicate a much greater degree of skeletal calcification. This has taken place in spite of the fact that he is now suffering from chronic glomerular nephritis with approximately the same degree of renal impairment that he showed during the study. The only difference in his present condition is that he is no longer in the nephrotic stage and now has a normal serum protein and calcium level. He has not reached the stage of advanced renal failure with serious phosphorus retention and acidosis.

ing the general picture of the disturbance of calcium metabolism in nephrosis: (1) normal growth of long bones and (2) apparently adequate calcification of the epiphyses.

The following interpretations have been made from these facts: (1) Bone matrix formation is normal. (2) Calcification of the shafts is incomplete because of a deficient supply of calcium. (3) Under conditions of general calcium deficit, the epiphyses of the growing bones are favored, with regard to calcium distribution, at the expense of the shafts.

SUMMARY AND CONCLUSIONS

I. The combination of generalized rarefaction of diaphyseal bone and relatively good calcification in the regions of epiphyseal growth has been a uniform x-ray finding in the children with nephrosis studied in this clinic. Most of these children have shown normal rates of bone growth and in none has there been any sign of rickets or other skeletal deformity.

II. The disturbances of calcium metabolism in nephrosis described by previous investigators have been found to be present also in the 3 nephrotic children who were the principal subjects of the present study. These disturbances are: (1) an abnormally low serum calcium concentration, (2) excretion of an abnormally great percentage of the ingested calcium in the stools, and (3) a virtual absence of calcium in the urine.

III. The excessive loss of calcium in the feces results in a rate of calcium storage far below normal for the growing child. Presumably this failure to store calcium at a normal rate leads to the skeletal decalcification demonstrable by x-ray. The presence of normal bone growth and relatively good epiphyseal calcification suggests that the available supply of calcium is distributed in the growing epiphyses in preference to the diaphyses.

IV. In one patient who underwent a sudden remission from his disease, the rapid disappearance of all clinical signs of nephrosis was accompanied by an equally prompt correction of the disturbances in calcium metabolism. This observation supports the view that the abnormal calcium metabolism is a part of the nephrotic syndrome.

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TABLE I

Comparison of plasma levels obtained using the Mindlin and Butler and Roe methods

Mindlin and Butler	Roe	Difference
0.2	0.3	+0.1
0.3	0.3	
0.3	0.5	+0.2
0.3	0.3	
0.4	0.3	-0.1
0.4	0.2	-0.2
0.5	0.6	+0.1
0.6	0.5	-0.1
0.8	0.8	
0.9	1.1	+0.2
0.9	0.7	-0.2
1.0	0.8	-0.2
1.0	1.0	
1.1	1.3	+0.2

A most important step in white layer determinations is the complete extraction of ascorbic acid from the cells. The cells should be allowed to stand in water for about one hour following laking and before protein precipitation. An alternative procedure is to freeze the cell suspension quickly, by standing the tube in dry ice. It was found that thawed cell suspensions, or those analyzed following one hour of soaking in water, yield values from 5 to 25 per cent higher than untreated cell suspensions.

When the cells have been extracted completely, there is no difference in the ascorbic acid levels of the platelet and leukocyte layers (2, 4). Specimens which include the lowest portion of the leukocyte layer, and which consequently contain many red cells, have been found to be about 40 per cent lower than the upper layer. For consistent results, care must be taken to avoid the introduction of red cells from the lowest portion of the white layer into the specimens. Hemolysis must be avoided throughout the collection and preparation of blood specimens, so that the white layer is clearly defined. A sample smaller than 5 mgm. is not applicable to this method. When a capillary pipette of about 0.9 mm. diameter is used to draw up the specimen, the lowest portion of the white layer is not disturbed, and the specimen

men used by Butler and Cushman. Duplicate plasma and whole blood determinations gave identical results, as did aliquot specimens of white layer suspensions. The method appears to be completely reliable for making single determinations, which is a distinct advantage in routine procedures.

The determination of the white layer ascorbic acid level by this method has the practical disadvantage that about 6 hours are required for the completion of the analysis. This is due to the 2½ hour period of centrifugation and the 3 hours of incubation required. It was found that the weighed white cells may be stored overnight in a vacuum bottle containing dry ice, either dry, in water suspension, or in water and trichloroacetic acid, with no loss of ascorbic acid. Whole blood and plasma may be treated similarly. Specimens may therefore be stored frozen until a convenient time for completion of the analysis.

B. COMPARISON OF THE LEVELS OF ASCORBIC ACID IN WHOLE BLOOD, PLASMA, RED CELLS, AND WHITE LAYER⁹

These studies include 167 comparative analyses for 99 individuals. With the exception of 29 studies on 17 adults who were members of the house and nursing staffs of the New York Hospital, all of whom were in apparent good health, the remaining 82 subjects, on whom 138 studies were made, were children between the ages of 2 and 14 years. Thirty-eight of these were ambulatory patients attending the out-patient pediatric clinics at the New York Hospital and 44 were convalescent non-febrile children on the pediatric pavilions. When several specimens of blood were taken from the same individual, the interval of time between specimens varied from days to weeks.

On the adult subjects and the pavilion patients, fasting morning blood specimens were taken. Specimens were taken from the clinic patients 2 to 3 hours following a breakfast which included no fruit of any kind nor tomato juice. All specimens were therefore fasting specimens or their equivalent with respect to vitamin C.

In an attempt to determine the relationship

⁹ We are indebted to the resident staff and the nursing service for their cooperation.

between the ascorbic acid levels in the various blood elements, plasma levels were compared to whole blood and red cell levels. In addition, a range of white layer levels in children was determined and the white layer levels were correlated with the plasma levels.

Comparison of whole blood and plasma levels

In 63 comparative determinations, it was found that the majority of the whole blood values were greater than the plasma values, with an average difference of 0.14 mgm. per cent. This difference between plasma and whole blood levels may be accounted for by the small contribution of the white cells in the whole blood specimen. The relation between plasma and whole blood values was not different at different levels, and the average whole blood levels parallel the curve of plasma levels. Since the experimental error in all available methods approximates 0.1 mgm. per cent, there would seem to be no advantage in making whole blood determinations in conjunction with or as a substitute for plasma determinations. The presence of a variable amount of red cells in dif-

ferent specimens did not appear to affect the whole blood values significantly (Figure 1).

Comparison of plasma and red cell levels

It has already been stated that the occasional development of turbidity during incubation may render the method used unreliable for red cell determinations. There were, however, 46 comparative determinations of red cell and plasma levels in which turbidity was absent. In these, it was observed that a majority of the red cell values were lower than the plasma values, with an average difference of 0.20 mgm. per cent. The average red cell values parallel the curve of plasma levels. This trend is consistent with other observations (2). The determination of red cell levels does not offer additional information with regard to tissue concentration of ascorbic acid, and the presence of red cells in the whole blood specimens may counterbalance the effect of the white cells in whole blood analyses (Figure 2).

Range in white layer levels

Butler and Cushman (4) reported a normal range in the white layer level of from 25 to 43

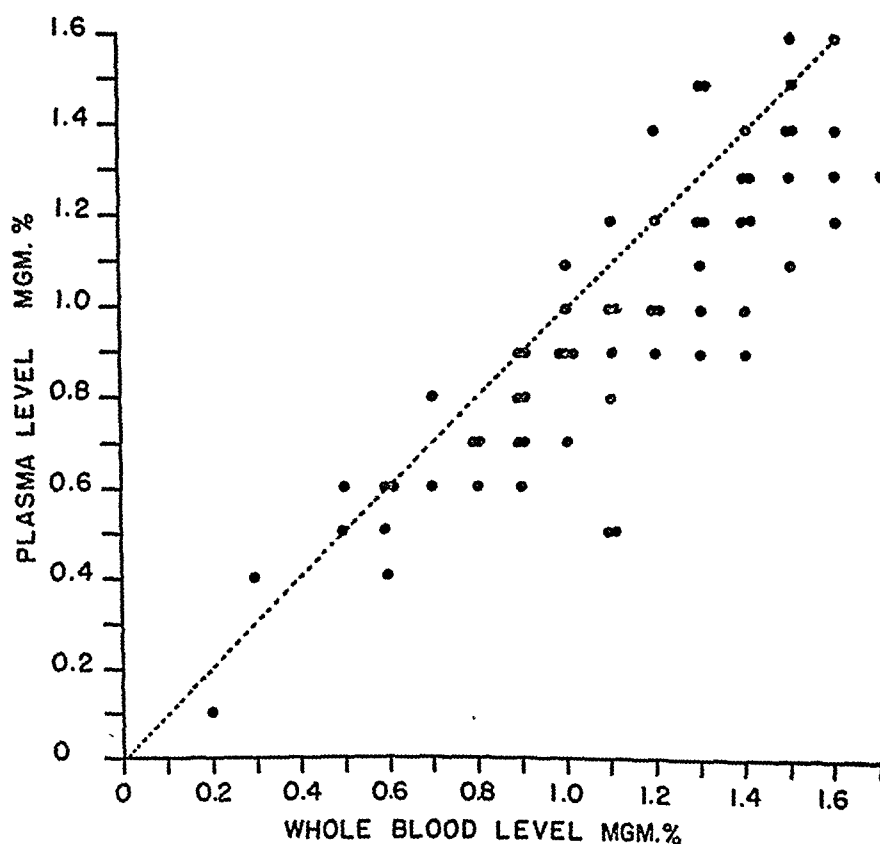


FIG. 1. COMPARISON OF WHOLE BLOOD AND PLASMA LEVELS OF ASCORBIC ACID

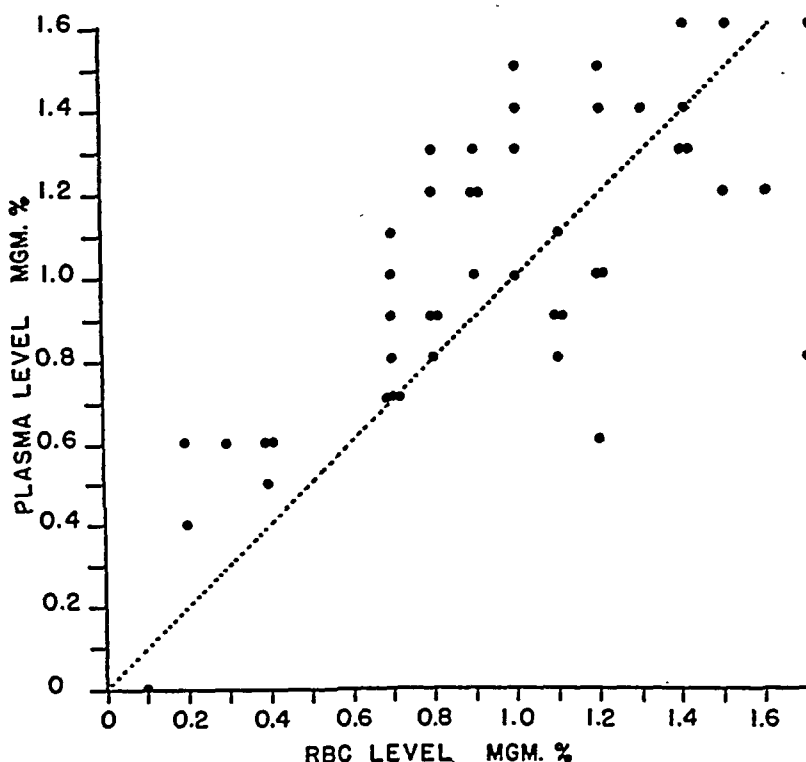


FIG. 2. COMPARISON OF RBC AND PLASMA LEVELS OF ASCORBIC ACID

mgm. per 100 grams leukocytes in a small series of determinations made on normal adults. The range found in 50 white layer determinations made on 48 children between the ages of 2 and 14 years extended from 6 to 58 mgm. per 100 grams leukocytes with the majority of the determinations falling between 11 and 30 mgm. per 100 grams leukocytes. Five determinations made on normal adults fell between 13 and 38 mgm. per 100 grams leukocytes.

Comparison of plasma and white layer levels

It was found that the majority of the children having plasma levels below 0.7 mgm. per cent had white layer levels in the lowest range. No subject having a plasma level below 0.7 mgm. per cent had a white layer level above 30. However, those having the highest plasma levels did not have the highest white layer levels, and at the lower plasma levels, there were relatively high white layer levels (Figure 3). Thus, as might be expected from previous studies (2 to 5), it appears that although there is a greater chance for the white layer level to be high if the plasma level is high, there is not a direct correlation.

Comment

Fasting specimens or those taken after a vitamin C-free breakfast were used throughout these studies. It was noted in 4 well nourished adult subjects, whose fasting plasma levels ranged from 0.5 to 0.9 mgm. per cent, that following a test dose of 100 mgm. of ascorbic acid, the plasma level rose 0.2 to 0.5 mgm. per cent, representing an increase of about 40 per cent in a 3-hour period. The white layer levels, which ranged from 15 to 35 mgm. per 100 grams leukocytes, increased 1 to 4 mgm., representing an increase of about 15 per cent. This would indicate that a fasting specimen is preferable for white layer determinations.

The correlation between dietary intake and ascorbic acid levels in the plasma and white layer is now under investigation. The range in white layer levels revealed in this study may be interpreted to reflect variable but probably low intakes in a non-febrile clinic population.

CONCLUSIONS

The method of Roe and Kuebler for determining the ascorbic acid content of whole blood and

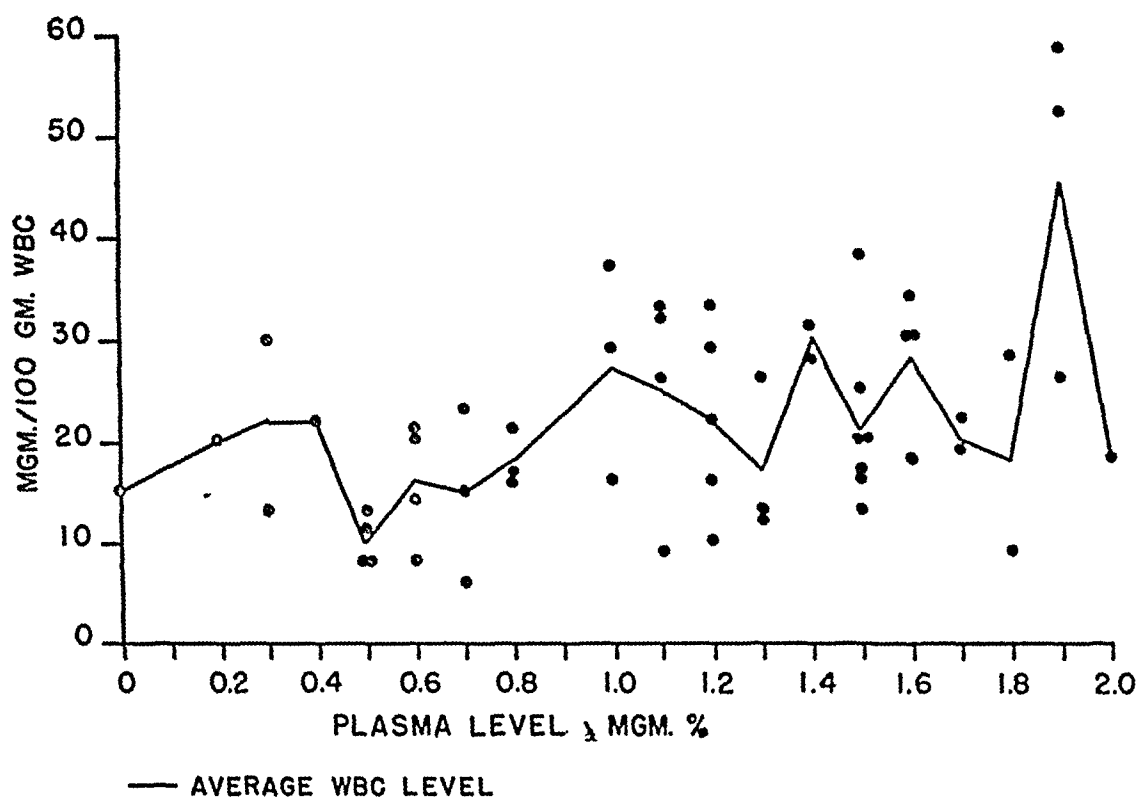


FIG. 3. WBC LEVELS AT DIFFERENT PLASMA LEVELS OF ASCORBIC ACID

plasma has been modified for routine use on small specimens of whole blood, plasma, and the white layer, the entire procedure requiring a single 4.5 ml. specimen of venous blood.

The determination of red cell levels by this method is not consistently reliable, due to occasional technical difficulty.

A comparison of whole blood, plasma, and red cell determinations made on specimens of fasting blood, indicates that the whole blood and red cell determinations provided no specific additional information beyond that gained by the plasma determinations.

The range in white layer levels in a series of ambulatory, non-febrile clinic children whose intake was unknown, but probably low, was found to be from 6 to 58 mgm. per 100 grams leukocytes, with the majority falling between 11 and 30.

No direct correlation was observed between the fasting plasma level and the white layer level. At high plasma levels, the highest and the lowest white layer levels were found.

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METABOLIC ALTERATIONS FOLLOWING THERMAL BURNS.¹

I. NITROGEN BALANCE IN EXPERIMENTAL BURNS

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Several investigators (1 to 4) have shown that a negative nitrogen balance exists following a burn. It was suggested that the prevention or alleviation of this loss of nitrogen might promote healing and improve the prognosis of the severely burned patient. Since, however, it is difficult to evaluate the significance of nitrogen balance studies in patients because of the marked variation in their age, their nutritional status previous to burning, and the depth and severity of their injury, it seemed advisable to carry out nitrogen balance studies in animals under controlled conditions.

METHODS AND MATERIALS

Adult female mongrel dogs were selected for the study. They were fed 10 or 11 grams per kilogram of body weight of the following mixture in parts per hundred: casein 37.4, dextrose 26.4, Crisco 22.2, salt mixture (U.S.P. Salt Mixture No. 1) 3.5, dried yeast 6.8, bone ash 2.7, and cod liver oil 1.0. The amount of food consumed daily by each animal was weighed, and samples of each batch of food were analyzed for nitrogen content. After the dogs had consumed this diet for a preliminary period of ten days to two weeks, they were placed in metabolism cages. Urine was collected under toluene and a daily aliquot removed and pooled for a 5-day period. At the beginning of the study, and at the end of each 5-day period, the bladder was catheterized to insure full collection. Feces, marked with carmine, were preserved with an acid-alcohol solution, dried on the steam bath and ground to a powder.

The nitrogen balance and body weight were followed for three 5-day periods to insure nitrogen equilibrium or a slightly positive balance and the maintenance of relatively constant weight. The animals were then burned under anesthesia (intravenous nembutal). Hot irons with a uniform surface area (12.57 cm.) were employed so that an estimate of the surface area burned could be calculated. A deep second or third degree burn was

produced, involving 20 or 30 per cent of the body surface (calculated from surface area (5)). Nitrogen balance studies were continued during the postburn period with the same food intake as that during the control period.

Total nitrogen was determined by the macro-Kjeldahl method. The ammonia was distilled into 75 ml. of saturated boric acid, and the mixture of bromocresol green and methyl red suggested by Ma and Zuazaga (6) was used as an indicator. Urea and ammonia nitrogen were determined by the method of Van Slyke and Cullen (7).

RESULTS

The results are summarized in Table I. Following the burn, all the animals showed a marked negative nitrogen balance on an intake of nitrogen that had been sufficient to maintain a positive balance in the periods previous to the burn. The nitrogen loss continued for approximately the same length of time (15 days) in all 4 dogs. A mild diarrhea was exhibited in all the animals after the burn. Dog 3 developed a severe diarrhea and showed a marked loss of appetite a week after the burn and died 11 days postburn. An increased nitrogen loss did not occur in the feces during the period of loose stools; only dog 7 showed a slightly higher fecal nitrogen output and diarrhea was not so marked in this dog as in animals 3 and 4.

Although an actual increase in all of the nitrogenous products in the urine occurred after burning, the percentage of nitrogen excreted as urea plus ammonia nitrogen was not markedly altered. In the 5-day period following the burn, dogs 7 and 8 showed no change, but in dogs 3 and 4, the percentage of total nitrogen excreted as urea and ammonia was decreased approximately 10 per cent.

Fifteen days from the time of burning, the animals began to retain nitrogen, but they did not regain their weight. Dogs 7 and 8 were kept on the experimental diet for several weeks after ex-

¹ The work described in this paper was done in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Wayne University. It was aided in part by a grant from the Theodore A. McGraw Fund for surgical research.

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TABLE I
The effect of experimental burns on nitrogen balance

Dog	Period †	Av. N intake per day	Av. Nitrogen Output per day				Av. N balance	Body weight
			Total urinary N	Urea + ammonia N	Fecal N	Total N		
3		grams	grams	per cent	grams	grams	grams	kgm.
	1	6.64	5.39	91	0.47	5.86	0.78	10.80
	2	6.41	4.80	90	0.48	5.28	1.13	10.91
	3	6.41	5.13	90	0.45	5.58	0.83	10.80
	Burned (25 per cent)							
	4	6.41	8.14					
	5	2.56*	7.42	80	0.64	8.78	-2.37	9.77
	6	0*			0.57	7.99	-5.43	8.41
						2.60**	-2.60	died
4								
	1	6.61	4.90	91	0.40	5.30	1.31	10.91
	2	6.38	5.16	89	0.54	5.70	0.68	10.91
	3	6.38	4.89	95	0.48	5.37	1.01	11.13
	Burned (25 per cent)							
	4	6.38	8.88	79	0.45	9.33	-2.95	10.11
	5	6.38	8.34	89	0.61	8.95	-2.57	9.77
	6	6.33	7.15	89	0.59	7.74	-1.41	9.09
	7	sample lost	4.43			4.84	1.49	9.55
	8	6.33	4.27	90	0.41	4.66	1.67	9.43
	9	6.33		89	0.39			
7								
	1	11.72	9.50	89	0.66	10.16	1.56	18.40
	2	11.80	9.85	89	0.79	10.64	1.16	18.51
	3	11.85	10.06	90	0.85	10.91	0.94	19.05
	Burned (20 per cent)							
	4	11.78	13.99	89	1.10	15.09	-3.31	18.18
	5	11.63	13.34	85	1.22	14.56	-2.93	17.28
	6	11.19	12.55	87	1.08	13.63	-2.44	16.35
	7	11.19	8.49	89	0.95	9.44	1.75	16.35
	8	11.72	8.17	89	0.77	8.94	2.78	16.35
	9	11.62	8.81	86	0.78	9.59	2.03	16.23
8								
	1	6.89	6.07	82	0.75	6.82	0.07	12.72
	2	7.07	5.78	87	0.52	6.30	0.77	13.18
	3	7.11	6.14	88	0.80	6.94	0.17	12.95
	Burned (20 per cent)							
	4	6.77	8.39	87	0.92	9.31	-2.54	12.14
	5	6.21	7.66	83	0.72	8.38	-2.17	10.91
	6	5.84	6.84	85	0.98	7.82	-1.98	10.56
	7	6.27	5.24	86	0.60	5.84	0.43	10.32
	8	8.39	6.20	89	0.73	6.93	1.46	10.00
	9	7.83	6.63	88	0.59	7.22	0.61	10.45
	10	6.89	5.02	89	0.73	5.75	1.14	10.67

† Each period represents an average of 5 days.
* Food intake reduced because of loss of appetite.
** Urine and feces pooled because of diarrhea.

lections were terminated, but there was no marked gain in weight until the food intake was increased.

DISCUSSION

It has been suggested (1) that the negative nitrogen balance following burns may be due, at least in part, to a reduced nitrogen and caloric intake. Since the loss of nitrogen in these animals occurred on an intake sufficient to maintain a positive nitrogen balance previous to burning, the nitrogen deficit would seem to indicate an accelerated catabolism. This view is in agreement with the studies on patients with typhoid fever (8), fractures (9), and burned patients (10).

The loss of nitrogen after burning was marked. Dogs 3, 4, 7, and 8 lost respectively 52.00, 34.65, 43.40, and 33.45 grams of nitrogen. In dog 4, the nitrogen loss was equivalent to 216 grams of protein, or in terms of muscle, to 1080 grams (calculated as 20 per cent protein). Since this dog weighed 10.91 kgm., 1080 grams represents approximately 10 per cent of the total body weight. If the nitrogen loss is calculated in terms of plasma containing 6 grams of protein per 100 ml., dog 4 lost the equivalent of 3600 ml. of plasma or roughly 4 times her normal plasma volume. Since nitrogen loss in the wounds is known to occur (11), the total nitrogen loss was even greater than has been indicated.

In this study, no attempt was made to determine the exact nature of the lost protein. During the control periods, the urea nitrogen averaged 80 to 85 per cent and the ammonia nitrogen 6 per cent of the total urinary nitrogen output. After the burn, however, dogs 3 and 4 showed a 10 per cent decrease in the percentage of total nitrogen excreted as urea; the percentage of ammonia nitrogen remained the same. This change was not seen in dogs 7 and 8. Total excretion of creatine and creatinine in dogs 3 and 4 showed an increase, but in terms of percentage of total urinary nitrogen, the values were similar to those of the control periods. The marked decrease in the percentage of nitrogen excreted as urea reported by others (12) was at no time encountered in this study, even in samples of urine taken at 24 and 48 hours following the burn. In experiments on burned calves, Glenn, Muus, and Drinker (13) reported increased values in the blood non-pro-

tein nitrogen. Their results showed that it is possible, at least under certain conditions, to demonstrate that creatine is released in the burned area. They found that "undetermined nitrogen" did not increase proportionately more than the total non-protein nitrogen except in one case. Whatever the nature of the loss of nitrogen, it is evident that there is an accelerated breakdown of protein following burns, and thus an increased requirement.

Several means of correcting the nitrogen loss suggest themselves, namely, feeding increased amounts of protein, increasing the nitrogen-sparing foods, particularly carbohydrate, and the administration of hormones known to have a nitrogen-retaining effect (anterior pituitary growth principle or testosterone). Shaffer and Coleman (14) have reported that a high carbohydrate diet with a liberal intake of protein can correct or diminish the nitrogen loss encountered during the febrile stage of typhoid fever. Cuthbertson's work (9) on patients with fractures indicates that it may not be possible to stem completely the loss of body tissue during the period of maximum tissue catabolism, but that it can be diminished by feeding large amounts of protein and carbohydrate. Even though the loss of nitrogen after burns cannot be entirely prevented, it is evident that the protein intake should be increased above the normal requirement after the catabolic phase has subsided. Such a conclusion seems justified, since our experimental animals failed to regain their body weight on an intake that had been adequate during the control period. A significant nitrogen retention in one normal man and in 3 patients with gastric carcinoma was reported (15) with the use of parenterally administered testosterone propionate.

The effect of this nitrogen loss and the treatment of it are now being studied in dogs under the same conditions as reported in this paper.

SUMMARY

Changes in nitrogen balance and body weight were studied in 4 adult female dogs before and after burning. For about 15 days following the burn, there was a marked deficit of nitrogen on an intake that had been sufficient to maintain nitrogen equilibrium and body weight previous to

the burn. No marked alteration occurred in the percentage of nitrogen excreted as urea and ammonia. The animals did not regain their weight, even though retention of nitrogen began, until the diet was increased.

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THE USE OF SODIUM p-AMINOHIPPURATE FOR THE FUNCTIONAL EVALUATION OF THE HUMAN KIDNEY¹

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The advantages of p-aminohippuric acid over diodrast for the measurement of the effective renal plasma flow and tubular excretory mass have been noted in a previous paper (1). The present report describes the determination of tubular excretory capacity by p-aminohippuric acid (Tm_{PAH}),² the quantitative relations between filtration rate, effective renal plasma flow, and Tm_{PAH} in 31 normal subjects, in 10 patients with essential hypertension and in 3 patients with glomerulonephritis, and presents a comparison of Tm_{PAH} with the equivalent diodrast measurement (Tm_D) in a number of these individuals.

The subjects were volunteers from the Third (New York University) Medical Division of Bellevue Hospital. Those considered normal were afebrile.

METHODS

The data were collected by two methods. The first, designated here as the multiple period method and identical with the method used in previous studies from this laboratory (2, 3), consists of the determination of Tm_D or Tm_{PAH} by 5 or more successive urine collection periods of about 10 minutes each. The following stock solutions are used in the preparation of the infusion fluid: inulin 10 per cent, mannitol 25 per cent, diodrast 35 per cent, sodium p-aminohippurate 20 per cent. For the measurement of effective renal plasma flow and glomerular filtration rate, a priming infusion of 80 ml. of mannitol or 30 ml. of inulin and 2.3 ml. of diodrast or 4 ml. of sodium p-aminohippurate is mixed in a sterile 250 ml. flask and administered intravenously at the rate of 20 ml. per minute. This is immediately followed by a

sustaining infusion consisting of 80 ml. of mannitol, 70 ml. of inulin, and 10 ml. of diodrast or 20 ml. of sodium p-aminohippurate, made up to a total volume of 500 ml. with normal saline and administered intravenously at the rate of 4 ml. per minute.

For the measurement of maximal tubular excretory capacity (Tm_D or Tm_{PAH}), the infusion is stopped and a priming injection is made into the infusion tubing of 30 ml. of diodrast or 60 ml. of sodium p-aminohippurate, administered over a period of 10 minutes. The residual infusion is reinforced by the addition of 0.18 ml. of diodrast or 0.43 ml. of sodium p-aminohippurate per ml. of infusion remaining in the flask, after which the infusion rate is restored to 4 ml. per minute. The methods of collecting blood and urine samples are described in the above references.

The second or single period method, described below, represents part of a composite renal function test recently introduced for general clinical use (4). This test is designed to measure maximal concentration power, effective renal plasma flow, filtration rate, and maximal tubular excretory capacity with a minimal number of technical procedures. No breakfast is given on the morning of the test. Between 9:00 and 9:30 a.m., the patient drinks 1 liter of water. At the end of this time, a venous blood sample (B_0) is taken for plasma blanks and hemocrit, and a priming infusion of 30 ml. of inulin or 80 ml. of mannitol and 3 ml. of sodium p-aminohippurate is administered intravenously from an infusion flask, over a period of about 2 minutes. The sustaining infusion, consisting of 42 ml. of inulin or 66 ml. of mannitol and 10 ml. of sodium p-aminohippurate, made up to 350 ml. with normal saline, is added to the infusion flask as soon as the priming infusion has reached the neck and is administered at the rate of 4 ml. per minute. Following a 20-minute discard period, the bladder is emptied by catheter and rinsed one or more times with about 20 ml. of saline, and a blood sample (B_1) is taken. Twenty minutes later the bladder is emptied and rinsed again, and a second blood sample (B_2) is drawn.

For Tm_{PAH} measurement, a priming dose of 60 ml. of 20 per cent sodium p-aminohippurate³ is injected into the

¹ Aided by a grant from the Commonwealth Fund.

² A sterile solution (20 per cent) of sodium p-aminohippurate in 50 ml. ampoules and Reagent grade p-aminohippuric acid were supplied through the courtesy of the Medical-Research Division of Sharp and Dohme, Philadelphia, Pa. We are indebted to Sharp and Dohme for making available substantial quantities of these materials for clinical as well as experimental trial.

³ In earlier tests, 200 ml. of 10 per cent sodium p-aminohippurate were infused over a period of about 30 minutes as a priming dose, but it was found that the smaller quantity was adequate and could be given in a shorter time.

TABLE 1

*Subjects with normal renal function. Multiple period method*In this and subsequent tables, all clearance and T_m values are corrected to 1.73 sq. m. body surface area.

Subject	Sex	Date	Plasma clearance (Man- nitol) C_M	T_{mD}		T_{mPAH}		T_{mPAH}/T_{mD}		$\frac{C_M}{T_{mD}}$	$\frac{C_M}{T_{mPAH}}$	Diagnosis
			ml. per minute	mgm. iodine per minute	mM per minute	mgm. per minute	mM per minute	mgm. PAH mgm. iodine	mM PAH mM iodine			
A. A.	F	4-20-42	115			69.2	0.357	1.74	2.27	3.07	1.69	Peptic ulcer
		4-24-42	122	39.8	0.157							
L. B.	M	5- 1-42	132			68.6	0.354				1.93	Sciatica
		5- 8-42	122	52.1	0.205			1.32	1.73	2.34		
A. P.	F	5-15-42	79.0			51.5	0.365				1.53	Chronic chole-
		5-20-42	72.9	37.3	0.147			1.38	2.48	1.95		cystitis
H. Mc.	F	5-18-42	119			87.9	0.453				1.35	Sciatica
		6- 1-42	111	51.5	0.203			1.71	2.23	2.16		
W. C.	M	5-22-42	81.6			64.4	0.332				1.27	Upper respiratory
		5-29-42	66.3	35.3	0.139			1.82	2.39	1.89		infection
J. H.	M	5-25-42	110			78.5	0.405				1.39	Streptococcal
		5-27-42	97.3	41.2	0.162			1.90	2.50	2.36		pharyngitis
B. M.	M	10-28-42	83.6	40.6	0.160					2.06		Cholecystitis
		11- 2-42	106			63.4	0.327	1.56	2.04		1.67	
E. T.	M	11-11-42	91.8			61.8	0.318				1.49	Osteoarthritis
		11-13-42	68.1	31.0	0.122			1.99	2.61	2.19		
M. R.	F	6- 2-43	99.0	39.0	0.154					2.54		Rheumatoid
		6- 4-43	108			73.7	0.380	1.89	2.47		1.47	arthritis
W. D.	M	6- 7-43	81.1	28.8	0.113					2.81		Sacro-iliac strain
		6-11-43	87.4			57.2	0.295	1.99	2.61		1.53	
<i>m</i>			97.6	39.7		67.6		1.73	2.33	2.33	1.53	
<i>σ</i>			17.8	7.18		10.0		0.24	0.29	0.32	0.21	
<i>σ/m</i> × 100			18.2	18.1		14.8		13.9	12.4	13.7	13.7	

infusion tubing, with the infusion stopped, the injection requiring about 10 minutes, and then 0.43 ml. of sodium p-aminohippurate are added to each ml. of the residual infusion, after which the rate of infusion is restored to 4 ml. per minute.

Twenty minutes from the end of the priming injection, the bladder is emptied and rinsed and a blood sample (B_s) is taken. Twenty minutes later, another blood sample (B_4) is taken and the second urine specimen (U_2) collected by rinsing. The concentrations of the essential substrates as determined in the separated plasma from the 4 blood samples are plotted on semilogarithmic paper against time and the mean plasma concentrations determined by interpolation to the middle of the 2 urine collection periods. These values, with the analytical data derived from the 2 urine samples, are used to calculate the effective renal plasma flow, filtration rate, and T_{mPAH} .

(T_{mD} measurements were not made by the single period method in this paper, but this procedure will fit into the single period method, the doses required being the same as those described above for the multiple period method.)

The above quantities of diodrast and sodium p-aminohippurate in the sustaining infusion are recommended for patients with normal or moderately depressed renal function, and must be proportionately reduced in patients with marked functional impairment. This is essential in the

determination of effective renal plasma flow, since elevation of the plasma level of diodrast iodine or p-aminohippuric acid above 3 and 4 mgm. per cent, respectively, may depress the renal extraction ratio.

Plasma is precipitated by cadmium sulfate and p-aminohippuric acid is determined as described elsewhere (1). Inulin is determined by a modification (4) of Harrison's method (5), diodrast by Alpert's method (6) and mannitol by the method of Smith, Finkelstein, and Smith (7). The p-aminohippuric acid plasma blank is determined additively, i.e., by the addition of a known amount of substrate to the B_0 filtrate (1). Inulin and mannitol plasma blanks are determined by adding known quantities of substrate to a sample of B_0 plasma, prior to treatment with yeast and precipitation.⁴ Equivalent aqueous dilu-

⁴ Re-examination of the inulin method, now in process, reveals that apparently fructose-free inulin (repeatedly recrystallized) is taken up to an appreciable extent by yeast and recoveries are therefore less than 100 per cent. This deficient recovery tends to be compensated by an inulinoid blank derived from the yeast itself.

Loss of inulin onto yeast is not a significant source of error if plasmas and urines are yeasted and precipitated in an identical manner. The appropriate blank to be deducted from the plasma may be determined additively in the B_0 filtrate, i.e., by diluting a small quantity of stand-

TABLE II
Subjects with normal renal function. Single period method

Subject	Sex	Date	Plasma clearance		T_{mPAH}	$\frac{C_{IN}}{C_{PAH}}$	$\frac{C_{IN}}{T_{mPAH}}$	$\frac{C_{PAH}}{T_{mPAH}}$	Diagnosis
			(Inulin) C_{IN}	(PAH) C_{PAH}					
			ml. per minute	ml. per minute	mgm. per minute				
J. M.	M	4-6-43	135		96.4		1.40		Upper respiratory infection
C. Mc.	M	4-9-43	88.5		104		0.854		Upper respiratory infection
D. D.	F	4-14-43	106		66.4		1.59		Pneumococcal pneumonia
S. L.	M	4-21-43	90.8	494	87.4	0.184	1.04	5.64	Chronic bronchitis
R. P.	F	5-6-43	115	543	84.3	0.213	1.36	6.44	Pneumococcal pneumonia
M. R.	F	5-10-43	97.8	606	68.6	0.162	1.42	6.83	Rheumatoid arthritis
J. Y.	M	5-11-43	130	748	94.9	0.174	1.37	7.88	Upper respiratory infection
A. F.	M	5-18-43	153	769	74.8	0.199	2.04	10.3	Rheumatic fever
C. O.	M	5-19-43	123	954	75.8	0.130	1.63	12.6	Upper respiratory infection
L. P.	F	5-25-43	101	536	85.4	0.188	1.18	6.27	Cholelithiasis
G. L.	M	5-26-43	103		82.2		1.25		Pneumococcal pneumonia
C. S.	M	11-3-43	136	708	78.6	0.192	1.73	9.00	Filariasis
J. H.	M	11-10-43	103	506	96.4	0.203	1.07	5.25	Duodenal ulcer
P. S.	F	11-15-43	99.7	675	76.8	0.148	1.30	8.79	Chronic alcoholism
A. L.	F	11-26-43	107	464	79.8	0.229	1.34	5.82	Primary atypical pneumonia
P. M.	M	12-3-43	114	696	77.2	0.164	1.48	9.02	Upper respiratory infection
P. E.	F	12-10-43	94.2	445	62.0	0.212	1.52	7.17	Hysteria
M. G.	M	12-27-43	99.8	508		0.196			Pneumococcal pneumonia
J. O.	M	12-29-43	98.7	765	90.7	0.130	1.09	8.44	Pneumococcal pneumonia
A. K.	F	1-13-44	96.8	529	62.1	0.183	1.56	8.51	Pneumococcal pneumonia
R. C.	M	1-24-44	139	539	93.7	0.257	1.48	5.75	B. Friedlander pneumonia
J. W.	M	4-24-44	143	681		0.211		8.03	Upper respiratory pneumonia
		4-27-44	141	709	88.3	0.199	1.59		
m			112	621	82.2*	0.187	1.41	7.74	
σ			15.5	133	11.1	0.03	0.27	1.86	
$\sigma/m \times 100$			13.8	21.4	13.5	16.0	19.1	24.1	

* The mean value of T_{mPAH} obtained by combining the observations in 31 subjects using the single (Table I) and multiple (Table II) period technic was 77.5 ± 12.9 .

tions of inulin or mannitol should be yeasted and precipitated and the resulting values deducted from the total B_0 recovery to obtain the appropriate B_0 correction. Urine samples diluted to a U/P ratio of approximately 1.0 are yeasted and precipitated in the same manner as plasmas.

The equation used for the calculation of T_m is that of Smith, Goldring, and Chasis (8),

$$T_m = UV - PC_{IX}FW = [(C_D/C_{IX}) - FW]PC_{IX}$$

where U and P are the concentrations of diodrast iodine (or p-aminohippuric acid) in mgm. per ml. of urine and plasma, respectively, V is the urine flow in ml. per minute, C_{IX} is the inulin (or mannitol) clearance, and C_D is the diodrast (or p-aminohippurate) clearance in ml. of plasma per minute. FW is taken as 0.73 for diodrast (8) and as 0.83 for p-aminohippuric acid (1).

During the administration of the priming dose of sodium p-aminohippurate, nearly all patients complained of a sensation of warmth which began in the lumbar region, the epigastrium, or the perineum and, at times, was felt over the entire body. The sensation persisted until 3 to 5 minutes after the injection had been completed. Despite

and inulin solution with the B_0 filtrate prepared without the addition of inulin. The same procedure is applicable to mannitol.

an intense subjective sensation of warmth, the rectal temperature remained normal and no change in skin color was visible, although in some individuals perspiration was noted. The pulse rate and blood pressure remained unchanged. The only other reactions were occasional headache or nausea which disappeared after cessation of the priming dose. Three out of 43 patients vomited and 2 had a formed bowel movement about 5 minutes after the end of the injection. In most instances, symptoms could be controlled or prevented by slowing the rate of injection and in no case was it necessary to discontinue administration of the drug.

RESULTS

Four sets of data are presented in the tables: a comparison of T_{mD} and T_{mPAH} in 10 normal subjects (i.e., convalescent patients who were free of immediate evidences or history of renal disease), these values being determined on separate occasions and each datum representing the mean of 5 or more successive urine collection periods (Table I); an extension of observations on T_{mPAH} in 22 normal subjects by the single period

TABLE III
Subjects with essential hypertension. Single period method

Subject	Sex	Date	Plasma clearance			Tm_D		Tm_{PAH}		Tm_{PAH}/Tm_D		$\frac{C_M}{C_D}$	$\frac{C_M}{C_{PAH}}$	$\frac{C_M}{Tm_D}$	$\frac{C_M}{Tm_{PAH}}$
			(Mannitol) C_M	(Dio-drast) C_D	(PAH) C_{PAH}										
			ml. per minute	ml. per minute	ml. per minute	mgm. iodine per minute	mM per minute	mgm. per minute	mM per minute	mgm. PAH mgm. iodine	mM PAH mM iodine				
C. M.	F	3-27-42	82.6					58.6	0.302						1.41
		4- 6-42	70.4			41.7	0.164			1.41	1.84			1.69	
A. W.	F	4- 8-42	74.2			24.4	0.096							3.01	
		4-17-42	57.8					55.0	0.283	2.25	2.94				1.01
		10- 4-42	66.4			27.3	0.107							2.43	
		10-14-42	64.8					52.3	0.269	1.91	2.51				1.24
C. B.	M	4-22-42	82.5					58.9	0.304						1.40
		4-27-42	70.7			29.2	0.115			2.02	2.64			2.42	
		1-22-43	66.8	309	334	36.1	0.142					0.215	0.199	1.85	
		1-29-43	79.2					65.0	0.335	1.80	2.36				1.22
J. W.	F	4-29-42	74.5					63.0	0.325						1.18
		5- 6-42	68.3			37.8	0.149			1.67	2.18			1.80	
R. S.	M	11-23-42	91.1	475		40.2	0.158					0.192		2.27	
		12- 2-42	98.4		570			67.2	0.346	1.67	2.19		0.173		1.46
C. B.	F	1-20-43	88.7			36.4	0.143							2.44	
		1-25-43	95.1					68.5	0.353	1.88	2.47				1.39
P. L.	F	1-27-43	83.2	453	424	45.1	0.177					0.184	0.196	1.85	
		2- 3-43	99.0					84.0	0.433	1.86	2.45				1.18
A. P.	M	2-19-43	96.5	437	415			65.2	0.336			0.221	0.233		1.48
		2-22-43	86.2	536	473	35.1	0.138			1.85	2.43	0.161	0.182	2.45	
J. O.	F	2-26-43	93.1	390	410			68.8	0.354			0.239	0.227		1.35
		3- 1-43	84.9	407	393	27.1	0.107			2.53	3.31	0.209	0.216	3.13	
P. C.	M	5-19-43	76.2	470	442			43.9	0.226			0.162	0.172		1.74
		5-21-43	74.4	422	446	26.6	0.105			1.65	2.15	0.176	0.167	2.80	

$$\begin{array}{rcl} m & 1.88 & 2.46 \\ \sigma & 0.25 & 0.33 \end{array}$$

$$\frac{\sigma}{m} \times 100 \quad 13.3 \quad 13.4$$

method (Table II); a comparison of Tm_D and Tm_{PAH} in 10 subjects with essential hypertension (Table III); and a similar comparison in 3 subjects with glomerulonephritis (Table IV).

The data obtained by the multiple (Table I) and single period (Table II) methods have been summarized separately and treated statistically.

The mean value of Tm_{PAH} obtained by the multiple period method (67.6) is lower than that obtained by the single period method (82.2). However, the first group is only half as large as the second, and the mean value of Tm_D in the first group (39.7 ± 7.2) is lower than that previously obtained for either males (51.8 ± 8.73) or females (42.6 ± 9.46) (3). This discrepancy appears to be attributable to chance distribution, since the ratio of C_M/Tm_D in this group (2.33) approaches the values previously reported for normal subjects (2.63 for men and 2.81 for women)

(3) and since the C_M (or C_{IN})/ Tm_{PAH} ratios in the 2 groups in the present study are nearly identical (1.53 and 1.41). We therefore infer that the difference in Tm_{PAH} in the multiple period (Table I) and single period (Table II) groups is not related to the differences in the methods of study, and we feel justified in combining the 2 groups in arriving at a mean, normal value of Tm_{PAH} . This value, as cited in Table II, is 77.5 ± 12.9 . Since the total number of subjects is too small to permit subdivision by sexes, the establishment of a mean value for each sex must await the collection of more data.

The ratio Tm_{PAH}/Tm_D , when calculated on a molar basis, averages (2.33 ± 0.32), a figure considerably higher than in the dog, in which species it has a value of approximately 1.0 (1). This species difference has no explanation at the present time. In the limited number of patients stud-

TABLE IV
 Subjects with glomerulonephritis. Multiple period method

Subject	Sex	Date	Plasma clearance			Tm_D		Tm_{PAH}		Tm_{PAH}/Tm_D		$\frac{C_M}{C_D}$	$\frac{C_M}{C_{PAH}}$	$\frac{C_M}{Tm_D}$	$\frac{C_M}{Tm_{PAH}}$
			(Mannitol) C_M	(Diodrast) C_D	(PAH) C_{PAH}										
			ml. per minute	ml. per minute	ml. per minute	mgm. iodine per minute	mM per minute	mgm. per minute	mM per minute	mgm. PAH per minute	mM PAH per minute				
F. T. ¹	M	1-6-43	42.3	138	182			24.8	0.128			0.307	0.233		1.71
		1-18-43	33.5	158	178	12.6	0.050			1.96	2.56	0.211	0.189	2.65	
J. V. ¹	M	2-1-43	112	499	609	44.8	0.176					0.225	0.185	2.51	
		2-5-43	127					82.6	0.426	1.84	2.42				1.53
		2-15-43	120	513	678	49.4	0.195			1.67	2.18	0.233	0.177	2.42	
M. S. ²	M	3-5-43	93.2	484	504	33.3	0.131					0.192	0.185	2.80	
		3-8-43	83.1	340	447			64.4	0.332	1.94	2.53	0.244	0.186		1.29 ²
			Inulin										$\frac{C_{IN}}{C_{PAH}}$		$\frac{C_{IN}}{Tm_{PAH}}$
M. M. ²	M	1-11-44	106		811			70.8				0.131			1.50
		1-17-44	106		1298			83.4				0.081			1.27
		2-3-44	104		692			84.0				0.150			1.23
I. S. ²	M	1-31-44	32.4		264			47.6				0.123			0.68
		2-8-44	20.8		128			13.4				0.162			1.55
		2-23-44	39.4		267			32.5				0.148			1.21
		3-8-44	44.4		279			32.1				0.159			1.38

¹ Chronic diffuse glomerulonephritis.² Acute diffuse glomerulonephritis.

ied here, this ratio remains unchanged in the presence of essential hypertension and glomerulonephritis, indicating that in these subjects the loss of capacity on the part of the renal tubules to excrete diodrast is accompanied by a parallel loss of capacity to excrete p-aminohippuric acid. Whether or not this relationship will hold at all stages and in all forms of renal disease remains to be determined.

SUMMARY

Procedures are presented for the determination of the effective renal plasma flow (C_{PAH}) and the maximal rate of tubular excretion (Tm_{PAH}) of p-aminohippuric acid.

The mean value of Tm_{PAH} in a group of 31 normal subjects is 77.5 ± 12.9 mgm. per minute per 1.73 square meters of body surface. Values are also given for Tm_{PAH} in patients with essential hypertension and in 3 patients with glomerulonephritis.

The molar ratio of the maximal rate of tubular excretion of p-aminohippuric acid to that of diodrast (Tm_{PAH}/Tm_D) is 2.35 ± 0.32 in 10 normal subjects. Practically the same ratio (2.46 ± 0.33) is found in 10 subjects with essential hyperten-

sion. In 1 patient with acute and 2 with chronic diffuse glomerulonephritis, the ratio appears to be similar.

The intravenous administration of relatively large doses of sterile solutions of sodium p-aminohippurate causes no disturbing symptoms if the rate of injection is approximately 6 ml. of a 20 per cent solution per minute.

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THE TREATMENT OF LOBAR PNEUMONIA WITH PENICILLIN¹

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This communication, which deals with the use of penicillin in the treatment of lobar pneumonia, and an accompanying one (1) describing the local treatment of pneumococcal empyema with penicillin, are extensions and elaborations of an earlier report (2) on the same subjects. The material embodied in the earlier article consisted of 46 cases of pneumonia and 8 cases of pneumococcal empyema. In the present report, 64 additional cases of pneumonia are included, and, in the accompanying article, 13 additional cases of empyema have been added, making total numbers of 110 pneumonias and 21 empyemas, respectively, that have been assembled for presentation.

The results obtained with the use of penicillin in pneumonia were derived from cases that were available for treatment during two successive years, consisting of the seasons of 1942-43 and 1943-44. Data for the two periods are given separately in Table I and are also consolidated as total results.

Since the study has been primarily concerned with cases of pneumococcal etiology, other cases of acute pulmonary infections in which the bacteriology was indeterminate and which, in addition, presented various clinical and roentgenological findings of ill-defined suppurative and non-suppurative pneumonitis, have been excluded from the present analysis.

Serological types of pneumococci

The incidence of infections caused by the serological types of pneumococci, Types I to VIII, which most frequently give rise to severe pneumonia, are comparable for the two periods recorded in Table I. In the 1942-43 series, 30 out

of 46 cases were due to the particular pneumococcal types mentioned and in the 1943-44 series 41 out of 64 cases were caused by the same types, the number in each instance representing approximately two-thirds of the total. The most definite difference in the type incidence during the two separate years is to be noted in the occurrence of 17 cases of Type II pneumococcus pneumonia during 1943-44 as contrasted with 6 similar cases in the preceding year.

The number of cases listed bacteriologically as "unclassified" refers to instances in which the sputum contained pneumococci but the organisms were not identified either by quellung reaction or by mouse inoculation as belonging to Types I to XXXII. The patients listed as having "no sputum" were selected for inclusion in the pneumococcal group of pneumonias on the basis of a characteristic history and classical clinical findings, including leukocytosis. It seems not unlikely that if the typing of sputum from these cases had been accomplished they would have distributed themselves proportionately among the usual types.

Bacteremia

The fact that the initial blood culture was positive in 40 (36.3 per cent) instances indicates that the cases selected for treatment were particularly suitable for assaying the effect of the therapy. The incidence of bacteremia in the 1943-44 period was somewhat higher (39 per cent) than in the preceding year (32.6 per cent). The difference is accounted for by the 17 cases of Type II pneumococcal infection encountered during 1943-44 of which 10 (58 per cent) yielded positive blood cultures.

Response to penicillin therapy. A definite response to treatment with penicillin has been arbitrarily defined as occurring when fever and accompanying symptomatology either rapidly disappeared during the first 12 to 24 hours or signifi-

¹ The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

TABLE I
Results obtained from the treatment of pneumonia with penicillin

Pneumo. Types	Cases of 1942-43 series					Cases of 1943-44 series					Combined results				
	No. cases	Positive blood culture	Response to penicillin			No. cases	Positive blood culture	Response to penicillin			No. cases	Positive blood culture	Response to penicillin		
			Definite	Indefinite	Died			Definite	Indefinite	Died			Definite	Indefinite	Died
I	11	4	10		1	6	3	6			17	7	16		1
II	6	3	6			17	10	16		1	23	13	22		1
III	1	1	1			5	1	4	1		6	2	5		
IV	1	1	1			2	1	2			3	2	3		
V	5	1	4	1		2	2	2			7	3	6	1	
VI						1	0		1		1	0		1	
VII	3	2	3			5	2	3	1	1	8	4	6	1	1
VIII	4	2	3		1	3	2	3			7	4	6		1
IX	1	0			1						1	0			1
X						1	1	1			1	1	1		
XI	2	0	2								2	0	2		
XII	1	0	1			3	1	2		1	4	1	3		1
XV	1	0	1			1	1	1			2	1	2		
XIX to XXIX	3	1	2	1		2	0	1	1		5	1	3	2	
Unclassified	4	0	3	1		7	1	6		1	11	1	9	1	1
No sputum	3	0	2	1		9	0	7	2		12	0	9	3	
Total	46	15	39	4	3	64	25	54	6	4	110	40	93	10	7

cantly and progressively defervesced during the first 2 days. In other cases for which penicillin was also of definite value but in which the effect was not reflected by rapid changes in the course of the temperature, significant improvement was evident by the clearing of bacteremia and by considerable alleviation of distressing symptomatology such as general intoxication, cyanosis, and labored respirations. The latter group usually comprised the most severe infections and in spite of the fact that fever did not rapidly abate, their recovery indicated particularly the value of penicillin in pneumonia.

Ninety-three (84.6 per cent) of the 110 patients were considered to have exhibited a definite response to treatment with penicillin. In 71 (76 per cent) of the 93, the rate of recovery was both complete and abrupt as reflected in the fact that their temperatures reached normal limits within the first day or two following the institution of treatment. Thirteen of the patients who responded in this manner had bacteremia on admission.

As stated in the previous article (2), the rapidity with which the temperature fell to normal ranges following penicillin therapy was often a striking phenomenon, the suppression of the infection not infrequently becoming evident within the first 12 to 15 hours.

In the remaining 22 (24 per cent) of the 93 patients who responded favorably to penicillin, fever persisted beyond the period of treatment. Twenty of the 22 in this group had bacteremia and many were admitted late in the disease. They either had complications referable to the pneumococcal infection itself or their pneumonia was superimposed on some other pre-existing disorder. In spite of these circumstances, their recovery from pneumonia was complete.

The acute complicating factors which delayed recovery in the latter group of 22 severe cases were as follows: empyema—2 cases, sterile pleural effusion—3 cases, purulent arthritis—2 cases, delayed resolution—3 cases, co-existing pulmonary diseases such as bronchiectasis, asthma, or possibly primary atypical pneumonia—6 cases, purpura—1 case, leukemia—1 case, gangrene of the foot—1 case, salpingitis—1 case, and unexplained fever of 10 days' duration—2 cases.

On the basis of our present experience, if a patient believed to have pneumococcal pneumonia fails to respond to penicillin therapy with a significant drop in fever by the third day of treatment, it has been found important to review again several aspects of the case such as the bacterial etiology, the possible development of some pneumococcal complication, or the possible presence

of an additional intercurrent disorder that may account for retardation of recovery.

The capacity of penicillin to eradicate *bacteremia* has been found to be one of the most striking effects. Of the forty cases (36.3 per cent) of the whole series which had bacteremia on admission, in every instance irrespective of the ultimate outcome, the second blood culture, taken usually on the following day, was sterile.

A limited number of observations have been made on the rapidity with which the circulating organisms were eradicated. In 5 cases, repeated blood cultures were taken at 3-hourly intervals after each intravenous injection of penicillin. The rapidity with which the cultures became sterile was as follows: in 2 instances, 3 hours after the first intravenous dose of 25,000 units of penicillin; in 2 instances, 3 hours after the second dose; and in 1 instance, 3 hours after the third dose. The progressive decrease in the number of organisms in the blood stream appears to indicate the bactericidal action of penicillin, *in vivo*, comparable to the findings observed *in vitro* (3).

In Table I, 10 of the recovered cases have been listed as exhibiting an indefinite response to penicillin therapy. None of the group had bacteremia and none of them were seriously ill. They pursued febrile courses for 7 to 14 days with only gradual improvement in spite of the administration of presumably adequate amounts of penicillin.

Complications such as pleurisy, bronchiectasis, or pulmonary fibrosis, which might account for the equivocal response, were suspected of being present but were not definitely established. The explanation of the failure of this group to respond remains, therefore, obscure.

Deaths. Seven patients who received penicillin therapy died (mortality rate 6.3 per cent). One of the patients of the 1943-44 series included in the fatal group was moribund on admission and died 7 hours later. A second patient, also of the 1943-44 series, was admitted to our wards with pneumococcal (Type VIII) meningitis which had developed in association with an earlier pneumonia that had been treated with sulfadiazine.

If the 2 cases of the 1943-44 series just mentioned are excluded, 5 deaths remain as a basis for estimating a mortality rate of 4.5 per cent.

Details of the 3 fatal cases of the 1942-43 series were given in the previous article (2). Of the

remaining 2 deaths among the 1943-44 series, the circumstances were as follows: one of the patients was a 65-year-old woman with an indeterminate history. She had pneumonia, bacteremia, and purulent arthritis due to pneumococcus, Type XII. Auricular fibrillation was present. She had received 145,000 units of penicillin before exitus. A blood culture taken on the second day was sterile. She died 48 hours after admission without responding to treatment.

The second recent fatal case was a 42-year-old white male who was admitted on the 4th day of pneumonia due to pneumococcus, Type II. Bacteremia was present. He received a total of 400,000 units within the first 9 hours, 100,000 units being given intravenously every 3 hours for 4 doses. The dosage which he received represented part of a study directed toward attempting to determine whether or not relatively massive doses of penicillin given over a short period of time would be as efficacious as small doses extended over several days. He received a total amount of penicillin greater than that employed according to usual procedures. On the day following admission, he appeared improved and a second blood culture was sterile. Consequently, subsequent treatment was temporarily withheld. On the following day, however, he became worse. Penicillin therapy was reinstituted but the patient died 6 hours later.

This patient, as will be subsequently pointed out in a discussion of dosage and duration of treatment, illustrates the importance of a continuation of treatment over several days in relation to day of disease on which treatment was first instituted rather than the total quantity of penicillin administered during a brief period in severely ill patients.

Cases with pneumococcal complications

Although additional studies of pneumococcal empyema will be the subject of a separate communication (1), 2 cases are included in the present series since they were first seen during the acute pneumonic phase of the disease. They were treated by injections of penicillin both intravenously and intramuscularly. Bacteremia, which was present in each instance on admission, cleared within the first 24 hours. They ultimately recovered.

Whether or not empyema developed in spite of the otherwise successful administration of penicillin is not clear. The acute pneumonic signs during the first few days of observation overshadowed the possible evidence of the presence of empyema. In view of the fact that the patients were admitted on the 5th and 8th days, respectively, of severe pneumonia, it seems possible that the suppurative infection of the pleura was present before therapy was begun. Up to the present time, in our experience, neither the intravenous nor intramuscular administration of penicillin has been found to be of value in the treatment of empyema after it has been established.

In the present series of cases, the incidence of 2 cases of empyema (1.8 per cent) among 110 cases of pneumonia is lower than the rate of 5 per cent which is usually given as representing the frequency of empyema as a complication of pneumonia before the modern forms of specific chemotherapy were adopted.

Four cases of *purulent arthritis* complicating acute bacteremic pneumonia were encountered in the present series. The arthritis was present on admission and was bilateral in each of the cases. Knees, ankles, or wrists, or combinations of these sets of joints, were affected.

Pneumococci, Types I (2 cases), VIII, and XII, were isolated from the sputum, blood, and the purulent articular exudates.

One of the cases, as mentioned previously, terminated fatally.

The remaining 3 recovered from the acute pneumonia. With respect to the arthritis, one of them recovered completely after several weeks of gradual defervescence; in another, in whom the acute articular infection was superimposed on an extensive pre-existing osteoarthritis, the joints returned to their earlier status; in the third patient, destruction of articular tissues occurred with permanent disablement which has required prolonged orthopedic treatment.

It is interesting to note that in each of the 3 cases which recovered, the arthritic exudate became sterile following the intravenous injection of penicillin. With specimens of exudate from 2 of them, tests *in vitro* were performed which demonstrated the presence of active penicillin that had diffused from the blood stream. Other investigators (4) have also recently reported that

penicillin administered intravenously was found to diffuse into inflamed joints.

The 3 patients in whom delayed resolution occurred were admitted to the hospital on the 7th, 8th, and 10th days of disease, respectively. Their illness was severe and was accompanied by bacteremia. Among the patients in general, however, delayed resolution has not been found to occur as a characteristic of pneumonia treated with penicillin.

Dosage of penicillin. In the previous report (2), a scheme of dosage was developed which was based on observations derived from experimentally planned trials with respect to quantity of penicillin per dose, frequency of dosage, and duration of treatment. The treatment which was outlined may be summarized as follows:

For cases of moderate severity, the injections for each day consisted of 10,000 units per dose given intramuscularly for 4 doses and the continuation of the procedure was conditioned by the day of disease on which treatment was begun. In patients admitted within 3 days of the onset, treatment was continued for at least 4 days; in patients admitted after the 3rd day of illness, 3 consecutive days of treatment often proved sufficient.

For cases severely ill, the first 2 doses consisted of 25,000 units, given intravenously at 3-hour intervals, followed, also at the same interval, by 2 doses of 10,000 units, given intramuscularly for the first day. When a favorable response was evident, the subsequent daily treatment consisted of 4 doses of 10,000 units each, given intramuscularly at 3-hour intervals for each day that treatment was continued.

The procedure as outlined obviates the necessity of giving injections of penicillin constantly throughout each 24-hour period since the remissions evoked by a series of 4 injections were found to endure for 20 to 24 hours after the last dose. However, in view of the fact that patients were often admitted during the evening or night the injections of the second day followed the treatments of the first day as a continuous period of 8 injections.

According to this regime the total amount of penicillin per case varied from 120,000 to 190,000 units and treatment lasted for 3 to 4 days.

In reviewing the dosage of the cases of the

1943-44 series, an analysis reveals that of 15 cases with bacteremia the average amount per case was 248,000 units and of 15 cases without bacteremia, the average amount per case was 175,000 units. The two factors which accounted for the recent increases in dosage were a greater number of intravenous injections (4 to 8 in sequence) to severe cases and a tendency to extend treatment beyond the third or fourth day when appreciable degrees of fever persisted. Analyzed objectively, no very clear cut advantage was demonstrable from the increased dosage except such advantage as might be referable to insuring sufficient therapy for the patients who were seriously ill.

In the previous communication (2), details of individual cases were given in which treatment with moderate amounts of penicillin (10,000 units every 3 hours for 4 doses) were injected for a single day only. Among the cases so treated in which the illness was relatively mild, the brief period of therapy proved sufficient. However, in other patients in whom therapy was suspended after the administration of the first 40,000 units, the primary remission, which lasted approximately 24 hours, was followed by a relapse requiring further treatment.

Additional observations have been made with regard to the effectiveness of a single day of treatment by administering large doses on the day of admission. One patient, treated in this manner, received on the first day a total of 300,000 units given intravenously in 3 individual doses of 100,000 units each at 3-hour intervals. The prompt fall in temperature and symptomatic improvement which followed endured for 30 hours. A sharp exacerbation of the infection then developed which was controlled when penicillin therapy was resumed.

A second case, similarly treated for a single day, was described among the fatal cases. As stated earlier, he received 400,000 units on the day of admission.

Although each of these patients received larger total amounts of penicillin than the average quantity given even to severe cases, the failure of the single day method emphasizes the greater importance of continuation of therapy rather than the size of individual doses. The role of immunity developed by the patient appears to be an impor-

tant factor in transforming the remission of infection induced by the drug into permanent cure.

On the basis of our additional experience, therefore, it seems likely that the earlier recommendations remain essentially valid as a basis for assaying the amount of penicillin necessary to suppress the infection. However, the method of handling unusually serious cases encountered late in the disease may invite the use of large initial doses (25,000 to 100,000 units intravenously for the first day or two) for the purpose of eradicating the bacteremia as rapidly as possible and thus preventing metastatic complications such as meningitis, pericarditis, or endocarditis from developing.

Infections caused by pneumococci resistant to sulfadiazine treated with penicillin. Among the patients included in the present study, the infections in 3 instances were proved by laboratory tests to be caused by pneumococci that possessed a high degree of resistance to the antibacterial action of sulfadiazine. The serological types of the infecting strains were Types I, VII, and VIII, respectively. Mice, infected with the strains, died following injections of 10 to 100 M.L.D. in spite of treatment with amounts of sulfadiazine that were, in tests with other strains of pneumococci of a similar type, sufficient to effect cure.

In each of the patients from whom the resistant strains were derived, appreciable blood levels of sulfadiazine resulting from the immediately previous treatment were present concomitantly with a bacteremia. Prompt improvement leading to recovery followed penicillin therapy in each case.

Since the patients did not respond to the initial treatment with sulfadiazine, their courses suggested that the infecting pneumococci may have been sulfonamide-fast from the beginning of the disease rather than having acquired the property during treatment.

Similar conditions to those just described were presented in an earlier article (5) in which 2 patients having bacteremic pneumonia due to sulfonamide-fast pneumococci were successfully treated with type specific antipneumococcus serum.

Cases in which penicillin therapy was replaced by the administration of sulfadiazine. In 8 of the 10 patients (listed in Table I) who exhibited no appreciable response to penicillin, sulfadiazine was subsequently administered during the febrile

period. In 7 of them the change in treatment was without effect. None of the patients were seriously ill. They all recovered gradually by lysis.

In one patient from whose sputum pneumococcus Type III was isolated, the change in treatment from penicillin, which seemed ineffectual, to sulfadiazine was followed by prompt recovery. Bacteremia was not present in the case.

Since the sulfadiazine was first administered on the 9th day of disease, the possibility that spontaneous crisis may have occurred coincident with the change in therapy requires consideration in interpreting the results.

The patient's strain was not tested for fastness to penicillin. In this connection, it may be stated that among 20 different strains of pneumococci derived from patients, none have been encountered which exhibited by laboratory tests an appreciable degree of resistance to penicillin. Up to the present time, a review of the literature fails to reveal the occurrence of penicillin-fast strains of pneumococci derived from patients.

Summary

This report contains the results obtained from the treatment of 110 cases of pneumococcal pneumonia with penicillin during the seasons of 1942-43 and 1943-44.

The total mortality (7 cases) was 6.3 per cent or, if corrected as indicated in the text of the article, 4.5 per cent.

Among the patients who recovered, the effectiveness of penicillin was deemed to be definite in 84.6 per cent (93 cases) and to be equivocal in 9.1 per cent (10 cases).

Bacteremia, which occurred in 40 of the patients (36.3 per cent), was successfully eliminated in each case and this result constitutes one of the

most striking effects of penicillin therapy in pneumonia.

With respect to dosage of penicillin, emphasis has been placed on extension of treatment over several days (3 to 5) depending on the day of disease on which therapy is begun.

Repetition of injections continuously throughout each 24 hours of treatment has not, except in seriously ill individuals, appeared necessary or advantageous.

The total amounts of penicillin employed have ranged from 120,000 to 200,000 units in cases of moderate severity, and from 150,000 to 400,000 units in serious cases, depending on the evidence of response noted in the first 24 to 48 hours of treatment.

In 3 instances, infections caused by sulfonamide-fast pneumococci were effectively treated with penicillin.

No toxic reactions referable to penicillin have been noted.

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THE USE OF PENICILLIN IN THE LOCAL TREATMENT OF PNEUMOCOCCAL EMPYEMA^{1,2}

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In a previous article (1), results were presented concerning the use of penicillin, injected intrapleurally, in the treatment of pneumococcal empyema. The method of treatment, its effect on the clinical course of the disease, and the final outcome were described in 8 patients. In 7 of them, the infection was eradicated and the suppurative process cleared completely without requiring more than repeated medical thoracenteses for drainage. In 1 patient, a child of 2 years of age, surgical drainage by rib resection was employed when a relapse of the infection occurred shortly after cessation of the first injections of penicillin.

It is the purpose of the present article to supplement the previous report by adding the results obtained from 13 additional cases of pneumococcal empyema, which have been successfully treated in a similar manner.

Pertinent features of the recent cases, hitherto unreported, will be described in the text of this article and, in addition, data from the total of 21 cases will be consolidated and discussed on the basis of the larger experience.

Patients used for study. In 19 of the patients, the empyema occurred in a typical manner as a complication of lobar pneumonia. In the remaining 2, the history suggested that the empyema had developed from a bronchopleural fistula which occurred in association with an acute pneumonitis.

Two of the patients had long-standing pulmo-

nary tuberculosis on which pneumonia and empyema were superimposed.

Only 2 of the patients came under our observation during the acute pneumonic phase of the infection. The remainder were transferred from other wards of Bellevue Hospital or elsewhere after the pneumonia had subsided and empyema had developed. The period in the infection at which penicillin therapy was first instituted varied from the 4th to the 48th day.

Neither intramuscular nor intravenous injections of penicillin were used in the treatment except in the 2 patients who had acute pneumonia and bacteremia at the time of admission. After the initial phase of active pneumonia was controlled by intravenous injections of penicillin, subsequent treatment was limited to intrapleural injections.

Selected data on the 21 patients are given in Table I. The patients who were described in a previous article (1) are designated by P (1P, 2P, 3P, 10P, 11P, 12P, 17P, and 19P). The recent cases are designated by numbers only (4, 5, 6, 7, 8, 9, 13, 14, 15, 16, 18, 20, and 21).

The discussions which follow center around the data presented in Table I.

The *ages* of the patients ranged from 2 to 71 years. Three of them, ages 2, 8, and 11 years, respectively, were treated on the Pediatric Service. The immediate response to treatment and the degree of ultimate clearing of the lesion were comparable in each of the age groups.

With regard to *sex*, it may be noted from Table I that there were 16 males and 5 females. The preponderance of one sex suggests an increased frequency of empyema among males although all of the factors that might account for the uneven occurrence have not been analyzed.

Among the *serological types of pneumococci* responsible for the empyemas, Type I infections 16

¹ This investigation was aided in part by the Commission on Pneumonia, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army.

² The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for clinical investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

cases) were the most frequent. The incidence is in consonance with previous reports that empyema is found as a complication of Type I pneumococcus pneumonia more often than in connection with other types.

In 14 (66 per cent) of the cases, pneumococci Types I to VIII, were found to be the causative agents. From the remaining 7 cases, pneumococci of the higher types ranging from Types XI to XX were identified.

The figures listed in Table I under *Day of disease* (Pneumonia) refer to the interval between the onset of the primary pneumonia from which empyema developed and the first thoracentesis at which penicillin was introduced. The data as given serve to indicate the approximate duration of infection before local therapy was instituted.

It may be noted from Table I that cases were first seen both early and late in the infection. However, there appeared to be no uniform relationship between the degree of effectiveness of treatment and the duration of infection before therapy was instituted. The cases treated late responded in a satisfactory manner and required no greater amount of treatment than early cases.

Mode of treatment. The solutions of penicillin were prepared with isotonic salt solution usually in the ratio of 1000 units per ml. When the amount of exudate removed by aspiration was insufficient to permit the introduction of the proper quantity of the solution, the planned dose of penicillin units was maintained by decreasing the amount of physiological salt solution used as solvent.

The 3 factors in the intrapleural therapy with penicillin which have received special consideration were the amount of penicillin per dose, frequency of injections, and duration of treatment.

Individual dosages in different cases have varied from as little as 5,000 units in the earliest cases to 100,000 units in others. Without elaborating unnecessary details, it has been concluded that less than 20,000 units per dose is liable to be insufficient and it was previously suggested (1) that 30,000 to 40,000 units appeared satisfactory. More recently, other cases have received 50,000 and 100,000 units per dose in the initial series of injections. The rate of clearing of the exudate and the speed of recuperation were not, however, significantly different from that of other cases

receiving the lesser amounts that were recommended. Although the optimal dosage for individual cases may vary, in view of the fact, as previously demonstrated (1), that penicillin is irritating locally in the pleural cavity, unnecessarily large doses may contribute to the transient reaccumulations of exudate and possibly delay final complete clearing without being more effective in eradicating the infection unless the extent of the pleural involvement is unusually large.

With respect to the frequency of intrapleural injections, in the previous study it was found, among the patients tested, that penicillin remained active for at least 48 hours after the initial injection. It was concluded, therefore, that in cases of pneumococcal infection no demonstrable advantage was derived from repetitions of injections oftener than on alternate days.

The number of repetitions of injections constituted the third factor in the therapeutic problem.

In 2 patients (M. W. (6) and M. B. (11P) of Table I) who had small empyemal pockets, only one injection was required, and in a third patient (A. Mc. (1P) of Table I), 2 doses were found to be adequate.

In the remaining 17 patients, the initial treatment consisted of 3 to 5 injections and in 11 of them, the first series of injections proved sufficient to effect complete eradication of the infection. In 7 others, however, relapses of the infection occurred when treatment was suspended, as evidenced by a return of organisms demonstrable in the pus and by a rise in fever.

The significant immediate effect of penicillin in all of the patients was regularly evident by the fact that in samples of exudate removed on the day following the first injection, the pneumococci were represented by disintegrating forms seen in direct smears, and cultures of the pus were in most instances sterile. Similar bacteriological observations on the second day failed to reveal viable or cultivable organisms.

However, the initial disappearance of pneumococci from the free pleural exudate occurred as promptly in the 7 instances of relapse as it did in the 14 other patients in whom the first series of injections proved adequate. Consequently, the arbitrary selection of the point at which therapy could be suspended has remained on an empirical basis since no specific clinical findings in the pa-

TABLE I

Data on patients with pneumococcal empyema treated by intrapleural injections of penicillin

Patient	Age	Sex	Type of pneumococcus	Day of disease (pneumonia)	Intrapleural penicillin			No. of aspirations	Days between 1st and last aspiration	Days in hospital (empyema)	Relapses
					No. of injections	Amount per dose, 1000's	Total, 1000's				
A. Mc. (1P)	57	M	I	33	2	40	80	5	21	42	None
J. D. (2P)	58	M	I	10	3	25	75	10	42	52	None
E. F. (3P)	11	F	I	34	3	25	75	3	13	21	None
R. C. (4)	8	M	I	48	4	30	120	6	21	See note	None
T. C. (5)	45	M	I	23	9 3 6	30 30-100	470	10	21	50	1
M. W. (6)	50	M	I	35	1	25	25	2	7	17	None
E. W. (7)	49	M	II	22	4 3 1	30 50	140	6	14	28	None
J. O'M. (8)	45	M	II	28	7	50	350	10	44	47	1
H. S. (9)	59	M	II	15	7 4 3	50 100	500	8	18	50	1
M. L. (10P)	42	F	V	7	9 3 6	5-10 20-30	155	16	44	79	3
M. B. (11P)	33	M	V	4	1	40	40	4	19	25	None
E. M. (12P)	35	M	VIII	15	5	15-25	100	8	47	62	None (3 pockets)
R. E. (13)	37	M	VIII	16	3	50	150	12	32	34	None
G. Q. (14)	49	M	VIII	12	9 8 1	50 100	400	11	32	42	1
R. M. (15)	23	F	XI	25	5	30	150	Tbc. See case comment.		45	None
L. T. (16)	38	F	XV	11	4	50	200	6	23	43	None
M. J. (17P)	2	M	XVI	18	3	5-15	30	3	Surgical	131	1
G. B. (18)	22	M	XVIII	6	4	50	200	Tbc. See case comment.		54	None
C. G. (19P)	27	F	XIX	45	3	30	90	3	11	26	1
A. S. (20)	50	M	XIX	11	3	30	90	3	8	33	None
F. E. (21)	71	M	XX	26	4 3 1	100 50	350	7	18	40	None

tients or laboratory examinations of the exudate have indicated the liability of recurrence of the infection. Further studies of this point are now in progress.

When the relapses occurred, treatment was re-instituted in 6 of the patients. Not infrequently, a larger amount of penicillin consisting of 50,000 or even 100,000 units per dose was injected. Irrespective of the size of the doses, the infection

diminished in each instance as promptly as it had done after the first series of injections and the return to treatment following the first relapse resulted in permanent cure.

The occurrence of 3 relapses in one patient (M. L. (10P) of Table I) was obviously related to the insufficiency of the early doses. It is interesting to note, however, that her ultimate recovery was as complete as that of any of the other

patients who were successfully treated in shorter periods of time. (X-ray photographs of the case were reproduced in the previous article (1).)

Among the 7 patients in whom recurrences of the infection developed, viable pneumococci became demonstrable between the fourth and seventh day after the last introduction of penicillin. The maintenance of sterility, therefore, as determined by direct examination of pus and by cultures, for as long as 8 days after cessation of therapy, has, in the present series of cases, been found to be a reliable indication of the complete eradication of the infecting organisms.

The data with respect to the number of repeated injections of penicillin that were employed may be summarized as follows: Among the 14 patients who recovered following the initial treatment,

- 2 received 1 injection of penicillin
- 1 received 2 injections of penicillin
- 4 received 3 injections of penicillin
- 5 received 4 injections of penicillin
- 2 received 5 injections of penicillin.

Of the 7 patients who had relapses of the infection—excluding 2 that received only 5,000 units per dose at the beginning of treatment—among the remaining 5, 3 received an initial series of 3 injections, and 2 received an initial series of 4 injections.

When the first period of therapy proved insufficient, the number of treatments that were subsequently employed ranged from 3 to 6 injections according to subjective estimates of individual factors presented by different cases.

Number of aspirations. The total number of aspirations listed in Table I refers to thoracenteses that were performed both during and after treatment. Each injection of penicillin was preceded by removal of as much exudate as possible. After penicillin therapy was discontinued, subsequent aspirations were carried out as indicated by the physical state of the patient and x-ray examinations of the chest.

The number of post-therapeutic aspirations of the chest varied from none in 4 cases to a maximum of 8 and 9, respectively, in 2 of the cases. The average for all the group ranged from 3 to 4.

Time required to effect recovery. Because of the gradual nature of the resolution of the lesion, the selection of a period representing the termina-

tion of the resolving process has proved to be unavoidably vague. However, in order to gain some information with respect to the duration of the illness under penicillin therapy, the first unsuccessful attempt at thoracentesis was selected as most appropriately indicating the essential end-point and the intervals between the *first and last successful aspirations* are listed in Table I. Utilizing the figures given in the column, the average duration of illness was found to be 24 days. The shortest periods were 7 and 8 days (M. W. (6) and A. S. (20) of Table I). The longest periods, 44, 44, and 47 days were required by 2 patients (J. O'M. (8) and M. L. (10P) of Table I) who had relapses of the infection and by one patient (E. M. (12P) of Table I) who developed 3 separate pockets.

Among the patients who did not have relapses, the average time was approximately 19 days.

There is also listed in Table I the number of days that the patients remained in the hospital after treatment was instituted. Since the patients were not infrequently kept in the hospital for purposes of observation particularly with reference to the rate of clearing and possibility of recurrence or the development of chronic empyema, the duration of their stay did not represent the period requiring hospital care. Even before the period of repeated aspirations ceased, they were ambulatory and were gaining weight. A low grade fever, 99° to 100° F., and slight leukocytosis, 9,000 to 10,000 W.B.C. per c.mm. were the chief general abnormal findings.

The evolution of the local pleural lesion followed an interesting course. The discrepancy between the relative slowness of the rate of disappearance of the exudate as contrasted with the rapidity of the sterilization of the pus bacteriologically, has been consistently observed. During the early stages of treatment, as previously noted, the rapid disintegration of the organisms and their disappearance constituted the most noticeable changes observed in samples of exudate obtained at daily intervals. During the later stages of the aspirations after the instillations of penicillin had ceased, the microscopical appearance of specimens of the exudate was characterized by a decrease in the number of leukocytes and changes in their morphology from the characteristic polymorphonuclear structure to swollen forms of disintegrat-

ing cells that stained poorly. Finally, there was almost complete absence of formed cellular elements. During the latter period, the character of the exudate did not, however, usually change to thin serous material but remained, in most instances, thick even during the period in which the lesion was regressing and the pleural shadow as seen by x-ray examination was decreasing in size and lessening in density.

Irrigation of the empyemal pockets with isotonic salt solution has been employed in an effort to hasten final clearing. In some instances, the procedure appeared to be advantageous and in others, the course was not significantly altered. In several of the patients, reaccumulations of exudate took place in the absence of any demonstrable bacteria and at a time when the general clinical condition of the patient was progressing in a satisfactory manner. The extent, therefore, to which repeated aspirations promote the ultimate clearing of the lesion is not clearly established. In this connection, the course of patient R. E. (No. 13 in Table I), which is given in "Comments on Individual Cases," is interesting to note.

By repeated x-ray examinations, both during hospitalization and after discharge, additional observations have been made on the progressive changes in the disease process that have occurred after the accumulation of free fluid had ceased and until the maximal degree of clearing appeared to be established.

Since the final outcome with respect to the anatomical and physiological state of the affected side of the thorax is the most important factor in estimating the value of therapy employed, a reduplication of pre-treatment x-ray photographs and also of the final examination are given for 7 of the recent series of 13 cases. (The 2 cases with tuberculosis are excluded since the tuberculosis involvement which has persisted overshadows the other changes.) Similar photographs of 6 of the earlier cases were presented in the previous article (1).

An examination of the photographs reveals that the final status in most of the cases consists of a partial flattening of the convex contour of the diaphragm and the extreme lateral corner of the pleural space is adherent. A diffuse thickening

of the whole area of pleural involvement is not generally evident, nor is there any decrease in the intercostal spaces of the affected side.

On physical examinations made at the time of the x-ray photographs, there was no appreciable limitation of expansion nor was deformity of the affected side noticeable. By fluoroscopy, the diaphragmatic excursions of the side previously involved were found in some of the cases to be limited but in none of them was the movement rigidly fixed. In a few instances, determinations of vital capacity were made and found to be normal.

It may be concluded, therefore, that the treatment which was employed has permitted the lungs of the affected side to return to a functional level that is essentially normal and that anatomically the thorax of the affected side is left with an amount of residual fibrosis that appears by x-ray examination to be considerably less than the area of the original empyemal involvement.

In a recent article, other investigators (2) have described the results which they obtained by the use of penicillin in acute empyema. In 7 of their cases, pneumococci were the causative bacteria, and streptococci of several varieties (hemolytic, non-hemolytic, anaerobic, and micro-aerophilic) were responsible for the infections in the others. Although a number of their patients recovered completely in an uneventful fashion, they concluded that "aspiration alone often leaves a thickened pleura and a rigid chest wall with the consequent disabilities of reduced vital capacity, fibrosis, and risk of recurrent pulmonary infection."

The patients described in this article have not exhibited the serious residual defects described above. Chronic empyema has not developed and convalescence has not been characterized by any of the debilitating effects of protracted infection. It should be emphasized that the present group of cases has been limited to pneumococcal infections. In a subsequent report, our experience with empyemata due to other species of pathogenic bacteria will be presented. Among the different types of suppurative pleurisy, differences in the character of the underlying lesion on which the empyemata were superimposed and variations in the necrotizing and other pathogenic properties of the several species of pleuropneumonia bacteria have been found to constitute factors of great importance

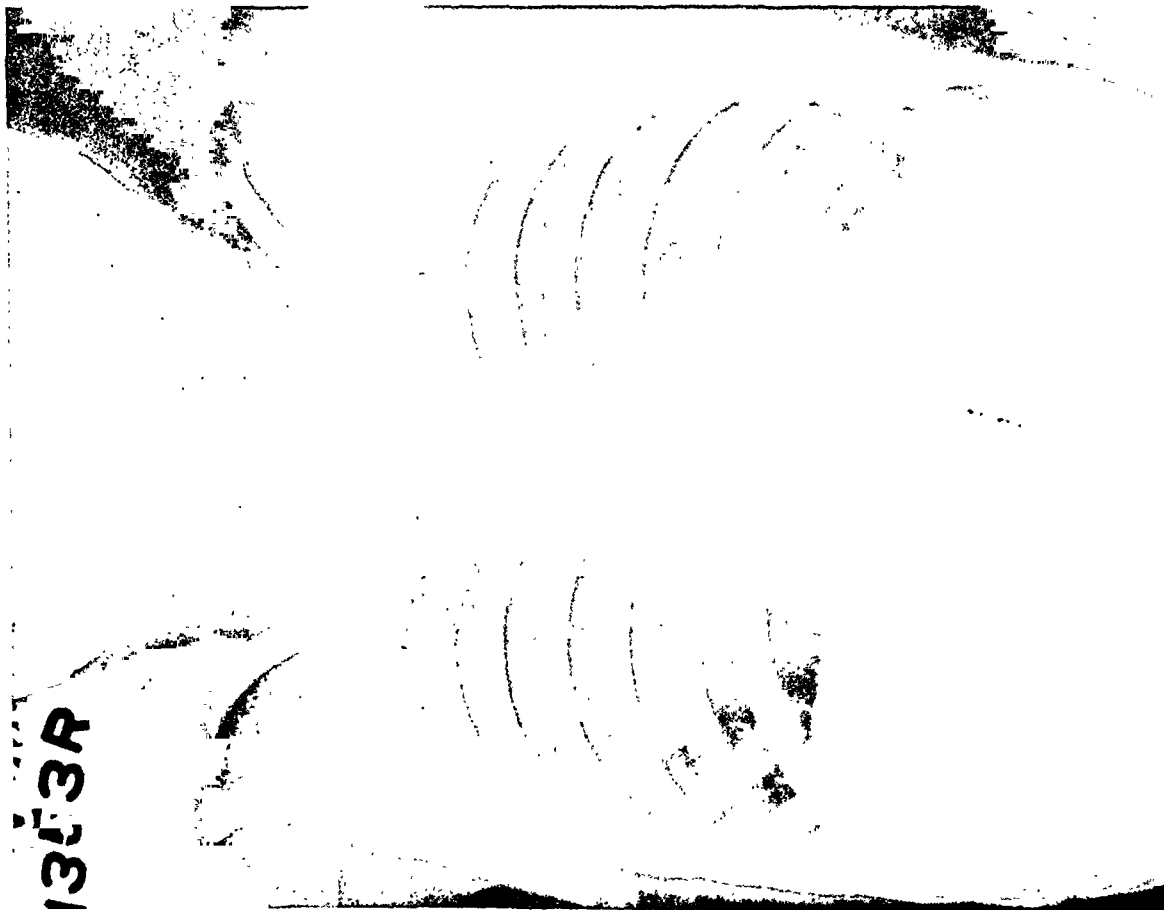


FIG. 2. CASE R. C. (4) 4 WEEKS AFTER CESSATION OF THERAPY



FIG. 1. CASE R. C. (4) BEFORE TREATMENT

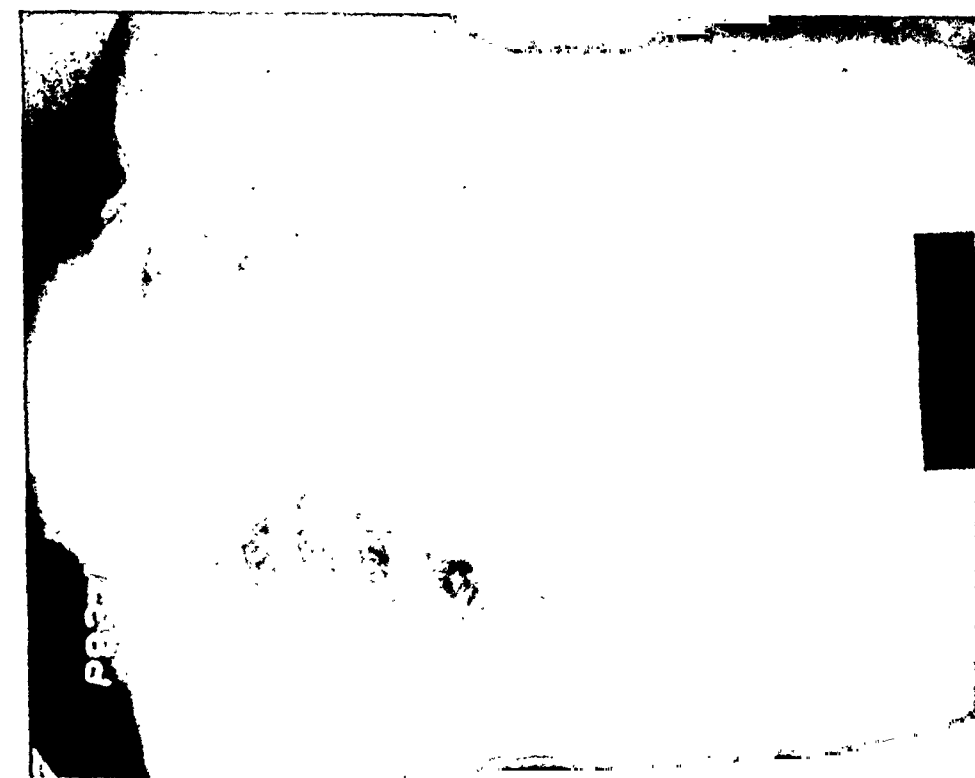


FIG. 3. CASE J. O'M. (8) BEFORE TREATMENT



FIG. 4. CASE J. O'M. (8) 3 1/2 MONTHS AFTER LEAVING HOSPITAL

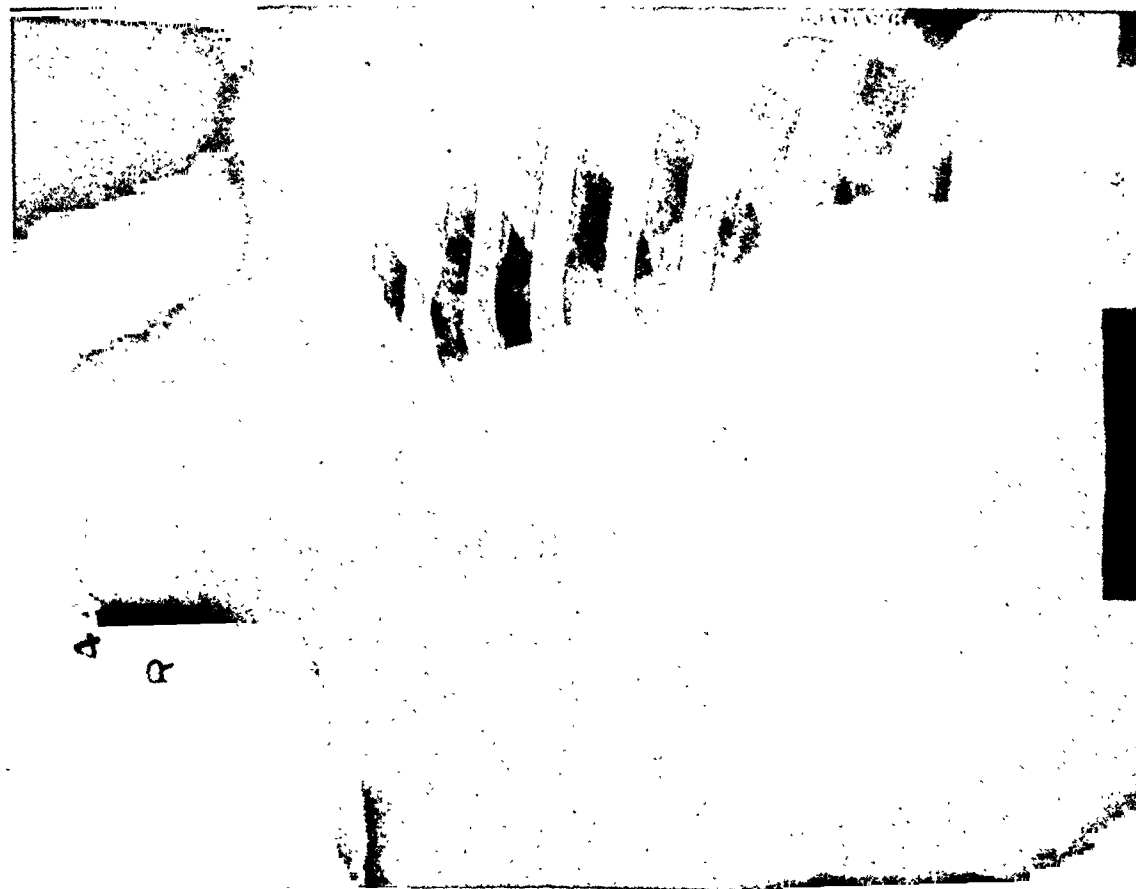


FIG. 5. CASE R. E. (13) BEFORE TREATMENT

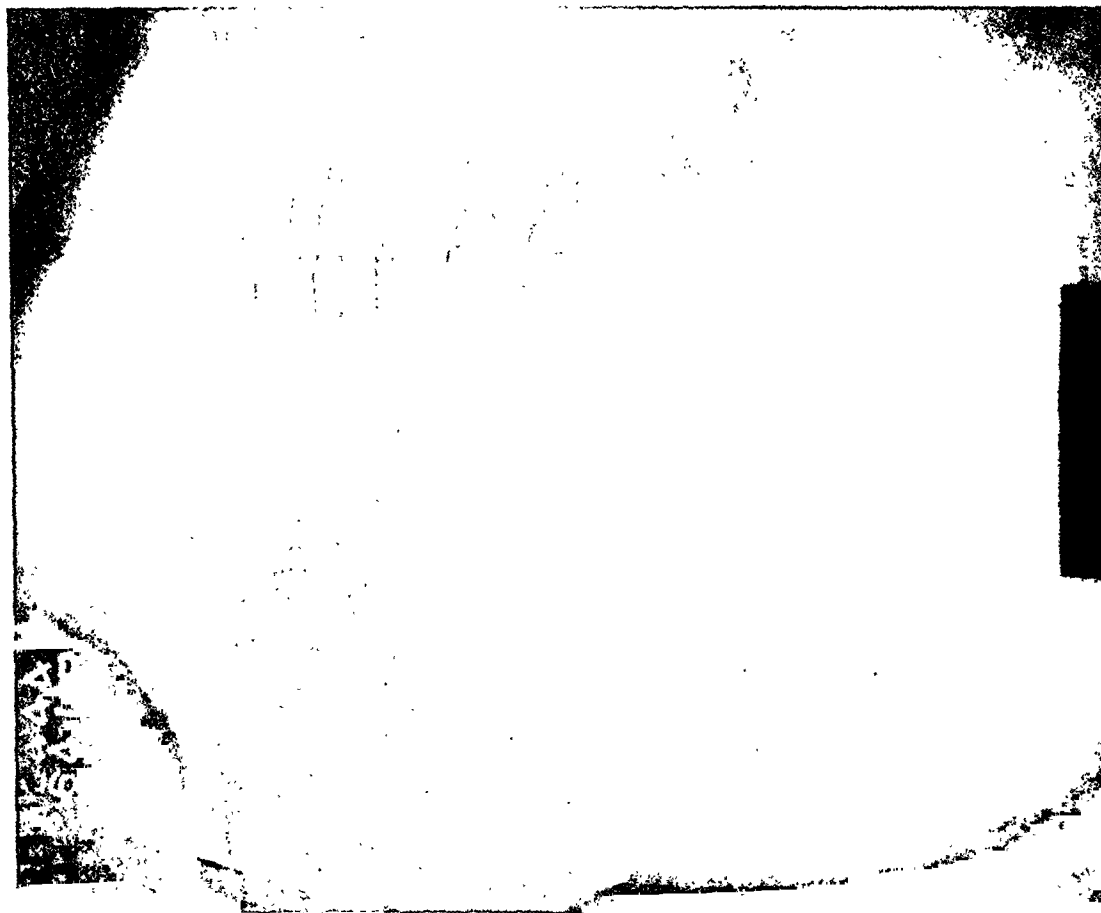


FIG. 6. CASE R. E. (13) 3 WEEKS AFTER CESSATION OF THERAPY
Photograph represents the extent of effusion that was present when the patient left the hospital against advice.



FIG. 7. CASE R. P. (33) 6 MONTHS AFTER LEAVING HOSPITAL. "Compensation of course under 'Comments on Individual Cases.'"



FIG. 8. CASE G. Q. (14) BEFORE TREATMENT

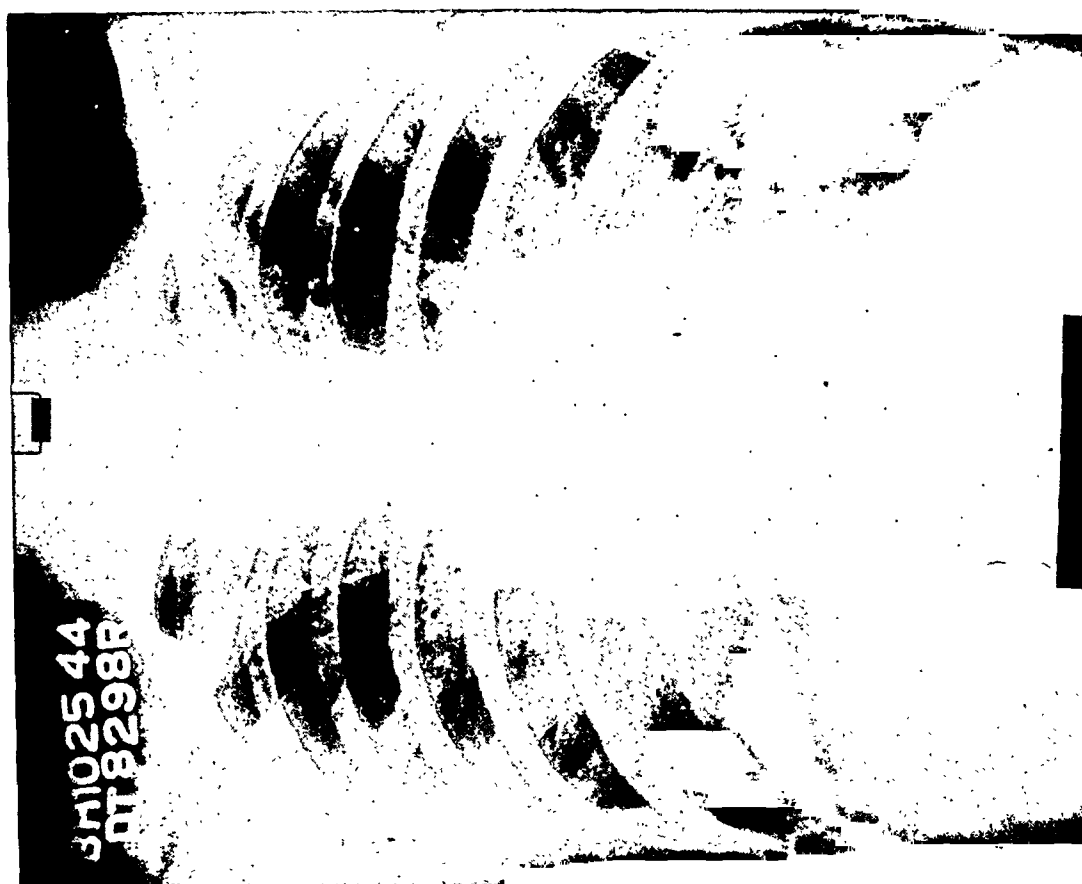


FIG. 9. CASE G. Q. (14) 4 MONTHS AFTER DISCHARGE
Continued clearing except for restricted layers of effusion or thickened
pleura in right lateral costophrenic angle.



FIG. 10. CASE L. T. (16) BEFORE TREATMENT

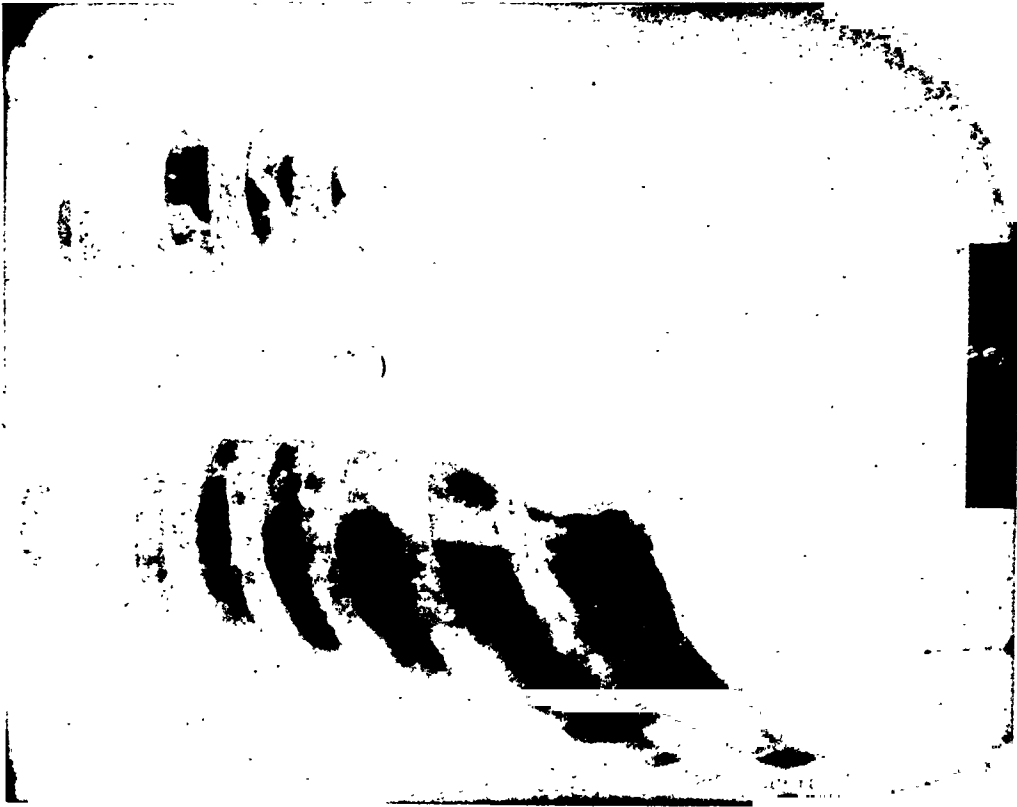


FIG. 12. CASE A. S. (20) BEFORE TREATMENT



FIG. 13. CASE A. S. (20) 160 DAYS AFTER LEAVING THE HOSPITAL



FIG. 13. CASE A. S. (20) 10 MONTHS AFTER LEAVING THE HOSPITAL.



FIG. 14. CASE F. E. (21) BEFORE TREATMENT

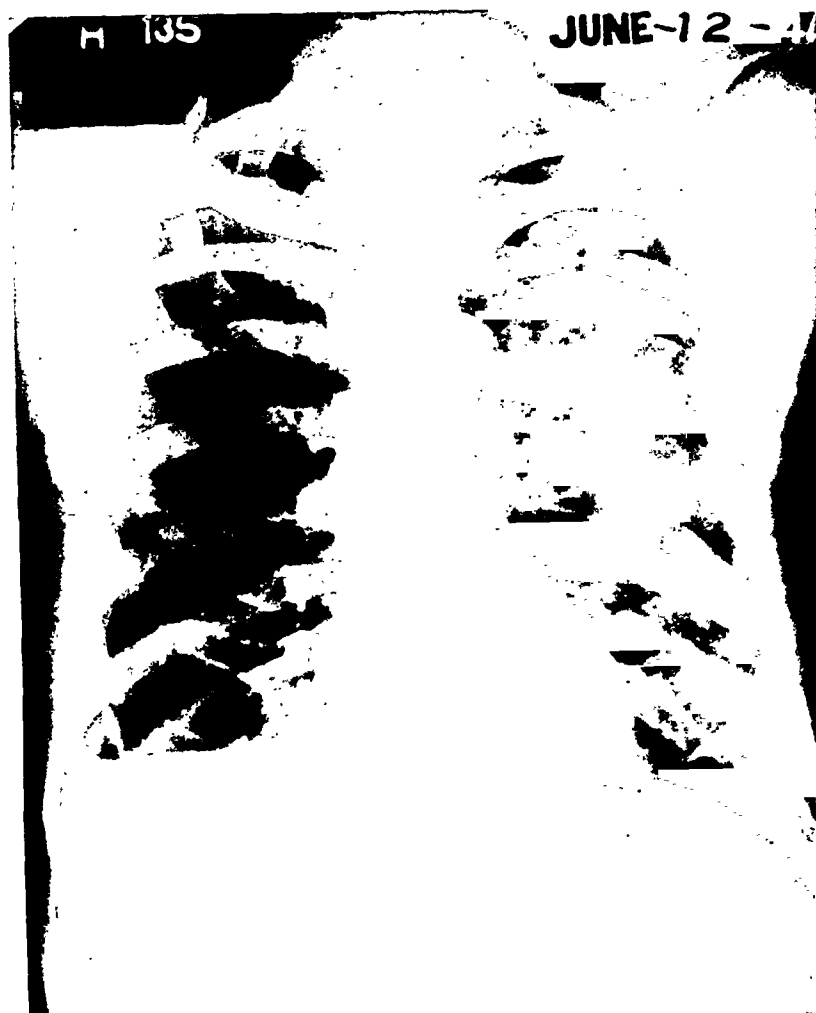


FIG. 15. CASE F. E. (21) ONE MONTH AFTER LEAVING THE HOSPITAL.*

* X-ray photograph of this figure was taken at the Home for Dependents, Welfare Island, N. Y., and is reproduced through the courtesy of the authorities of the Hospital (Dr. Maxwell Lewis, Supt.).

taken into account in formulating the most favorable therapeutic procedure with respect to the usefulness or limitations of penicillin. The findings recorded in this article suggest that exudates (pneumococcal) sterilized by penicillin and contained within a closed serous cavity present circumstances which are in many respects unique.

A tentative analysis of some of the special factors may be outlined as follows: When the viable pneumococci are destroyed by penicillin, the stimulus for the migration of leukocytes is considerably lessened and the remaining sterile exudate gradually assumes an acellular character. With the marked reduction in leukocytes, the resolving

process is, in part, deprived of one of its important constituents (proteolytic enzymes). The gross appearance of the exudate accordingly changes only to a limited degree as the clearing of the lesion gradually progresses.

The somewhat protracted rate of absorption from the pleural cavity appears to be dependent upon factors involving the degree of constraint or pressure of the exudate in relation to its relatively high concentration of protein within the pleural cavity. Alterations in the alveolar surface of the affected pleura that are dependent on the presence of an exudative process may also be a consideration.

Finally, the limitations in the development of fibrous tissue at the site of the infection suggest that the stimuli for fibroblastic reproduction derived from sterile exudates of pneumococcal origin are restricted in degree by comparison with the effect of certain other infections that are characterized by extensive local tissue necrosis.

Specific experimental studies concerning the problems just mentioned are now in progress.

COMMENTS ON INDIVIDUAL CASES

Two of the patients have had subsequent attacks of lobar pneumonia due to pneumococci of a serological type different from that causing the initial pneumonia and empyema. The infection involved the same lobe as that of the first infection. Empyema did not develop.

Case T. C. (No. 5 in Table I). The first attack which was complicated by empyema consisted of pneumonia and bacteremia due to pneumococcus, Type I. The second attack of pneumonia occurred while the patient was still in the hospital in the latter convalescent stages of empyema. There was a sudden rise in fever with thoracic pain and rusty sputum. Pneumococcus, Type III, was isolated from his sputum. No bacteremia was present. The response to intravenous injections of penicillin was prompt. There was no reaccumulation of pleural effusion, although the lobar involvement was the same in each attack.

Case A. S. (No. 20 in Table I). The first attack of pneumonia with which empyema was associated was caused by pneumococcus, Type XIX. He was readmitted to the hospital 5 months later with pneumonia and bacteremia, pneumococcus, Type I, and delirium tremens following an alcoholic spree. Recovery was satisfactory following intravenous penicillin therapy. No effusion developed in the affected side.

A case which later developed osteomyelitis

Case R. C. (No. 4 in Table I). The patient was an 8-year-old boy whose empyema was caused by pneumococcus, Type I. Just before the time for discharge from the hospital, he suddenly developed a high fever which proved to be due to osteomyelitis of the right femur. Pneumococci were not isolated from the blood or infected bone. The case had the usual characteristics of acute staphylococcal osteomyelitis. Surgical drainage and penicillin therapy were employed. There was no involvement of lungs or pleura.

Two patients had pulmonary tuberculosis

Case R. M. (No. 15 in Table I). Female, age 23 years. Tuberculosis had been present for 9 years. There had been wide spread involvement of both lungs. Previous x-rays had revealed extensive calcification involving particularly the right pleura, which was the site of the pneumococcal empyema. At the time she developed pneu-

monia, the tuberculosis was well controlled although not entirely inactive.

In the treatment of her pneumococcal empyema, she received a total of 5 injections of penicillin. (One dose of 25,000 units and 4 doses of 30,000 units.) Pneumococci were not recovered from any of the samples of exudate taken after the first dose of penicillin had been injected. The clinical course of the empyema was not different from that of the other patients, except that a thin layer of serous effusion has persisted from which tubercle bacilli have been recovered. Her general condition is considered to be good.

Case G. B. (No. 18 in Table I). Colored, male, age 22 years. The patient has had pulmonary tuberculosis for 6 years. During the past 2 years, he has been treated with artificial pneumothorax, repeatedly induced.

He developed pneumonia at a time when pneumothorax was present. Pyopneumothorax ensued due to pneumococcus, Type XVIII.

Pneumococci have not been recovered from the pleural effusion at any time since the first injection of penicillin. He received 4 injections of penicillin of 50,000 units each.

The pneumothorax persisted and the effusion continued to reform although its character gradually changed over a period of 6 weeks from thick, purulent material to a somewhat thinner sanguinous effusion.

In view of the fact that the partially collapsed tuberculous lung was unable to expand, a thoracoplasty was performed 3 months after the pneumococcal empyema had subsided.

A case complicated by marked arteriosclerosis and senility

Case F. E. (No. 21 in Table I). White, male, age 71 years. There was widespread arteriosclerosis and cerebral degeneration. The patient was disoriented and uncooperative.

His course did not differ from that of the others except that an intermittent fever persisted for 18 days after treatment was stopped. When he became afebrile he was transferred to another institution for custodial care. His final x-ray which shows almost complete clearing is presented.

In two cases the empyema was associated with a broncho-pleural fistula

Case M. W. (No. 6 in Table I). White, male, age 50 years. Before admission, the patient had suffered from an acute respiratory infection for 5 weeks. Because of the concomitant occurrence of copious purulent sputum and a thick pleural effusion, he was considered to have had a broncho-pleural fistula. On admission, he was not acutely ill, his temperature ranged from 99° to 100° F. and the broncho-pleural opening had sealed off as indicated by the absence of cough or sputum.

A small pocket containing 15 ml. of thick pus was found which contained Type I pneumococci.

A single injection of 25,000 units of penicillin proved sufficient to eradicate the infection and on only one subse-

quent occasion could fluid be obtained by aspiration. The patient progressed to complete recovery.

Case C. G. (No. 19P in Table I). White, female, age 27 years. This patient was reported in detail in the former article (1). Her diagnosis was pyopneumothorax. Pneumococcus, Type XIX, and *H. influenzae* were isolated from the pus.

The pyothorax cleared following intrapleural therapy with penicillin. However, the pneumothorax persisted and increased so that there was almost complete collapse of the right lung. She was discharged from the hospital afebrile and has been observed for the past year both on our Service and on the Chest Service of Bellevue Hospital.

Her right lung has gradually expanded until it has reached the thoracic wall except at the site of a small circumscribed effusion. Because of the excellence of the patient's general health no attempt has been made to aspirate the small effusion.

A patient who left the hospital while a large sterile pleural effusion was still present

Case R. E. (No. 13 in Table I). Colored, male, age 37 years. Intrapleural penicillin therapy was begun on the 16th day following the beginning of pneumonia due to pneumococcus, Type VIII. He received 50,000 units on each of 3 alternate days. Cultures were sterile after the first treatment.

An interesting feature of this case was the reaccumulation of large amounts of sterile pus. On 8 occasions after treatment was discontinued, amounts of exudate ranging from 250 to 375 ml. were drained off by thoracentesis.

His general health was very good during the latter period, he was ambulatory, and gained 10 lbs. in weight in 3 weeks.

He left the hospital against advice on the 34th hospital day while an effusion of considerable size was still present. He was seen subsequently 6 months after discharge. According to his history, he had resumed work as a janitor a few days after going home and had worked continuously.

An x-ray taken after the 6 months' interval (see x-ray) showed limited lateral thickening of the pleura without any residue of effusion being present.

In retrospect, the satisfactory course which this patient pursued brings into question the advantages that may be derived from repetitions of the aspirations after sterilization has been accomplished.

SUMMARY

The results obtained in the treatment of 21 cases of pneumococcal empyema by intrapleural injection of penicillin may be summarized as follows:

Twenty of the cases recovered without evidence of residual chronic infection or thoracic deformity or a detectable reduction in pulmonary function.

In 14 of them, a single series of local injections ranging from 1 to 5 doses given on alternate days proved sufficient.

In 7, relapses occurred when the initial course of treatment was suspended.

The reinstitution of local treatment with penicillin resulted in permanent clearing of the infection in 6 of the cases with recurrence.

Although the recurrences protracted the duration of the illness, the completeness of ultimate recovery was not impaired. The single unsuccessful case was a child of 2 years of age who was operated upon when the early treatment was followed by a relapse.

Two patients had low-grade active pulmonary tuberculosis of long standing on which pneumonia and empyema were superimposed. The pneumococcal infection of the pleura responded to the treatment in a manner similar to the other patients but the tuberculous infection remained unimproved.

The patients as a group have been followed in most instances for several months after discharge from the hospital. The clearing of the sterile deposits of exudation on the pleural surfaces, as determined by the x-ray examinations, required from 3 to 9 weeks. However, the period during which it was possible to obtain free fluid by aspiration was considerably shorter. Adopting as a measure of the duration of disease under penicillin therapy, the interval between the first and last successful thoracenteses, the average time was 24 days.

The general health of the patients during the post-therapeutic period of resolution was satisfactory. The usual manifestations of suppuration improved considerably in advance of the actual disappearance of the sterilized exudate.

The ultimate outcome has been characterized by the return of the affected side to essentially a normal functional state. The residual anatomical abnormalities, as illustrated by the accompanying x-ray photographs, indicates the limited degree of pleural thickening which has remained.

The method of treatment has, for the most part, consisted of injections of from 25,000 to 50,000 units of penicillin on alternate days for a minimum of 3 doses. The amount of the individual doses of penicillin and the duration of treatment

have been varied from the prescribed course according to special circumstances presented by individual cases with respect to extent of the involvement and the general condition of the patient.

Special factors that appear to be involved in the process of recovery have been discussed.

Two of the patients in the Pediatric age group (Patients E. F. (3P) and M. J. (17P) of Table I) were treated with the permission of Dr. James Wilson, Director of the Pediatric Service of Bellevue Hospital. The third pediatric patient (Patient R. C. (4) of Table I) was treated with the permission of Dr. C. W. Lester

and Dr. Fenwick Beekman, Chief of the Surgical Pediatric Service of Bellevue Hospital.

Data with regard to the pre- and post-empyema course of the 2 patients with tuberculosis are presented with the permission of Dr. J. B. Amberson, Director of the Chest Service of Bellevue Hospital.

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THE EFFECT OF CONTINUOUS INTRAVENOUS ADMINISTRATION OF HISTAMINE ON BASAL METABOLISM IN HUMAN BEINGS

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INTRODUCTION

In 1922, it was reported that histamine, repeatedly injected subcutaneously into rats for several days, produced little or no effect on metabolism (1). Tests were not made immediately after the subcutaneous injection, the earliest being 1½ hours after the injection of 20 mgm. Only in large doses was there a slight change in metabolism. On the basis of experiments, it was concluded that histamine does not belong to that group of substances which plays a major rôle in metabolic processes.

In 1928, it was found that a 1 per cent solution of histamine injected subcutaneously in human subjects obtained an 8 to 15 per cent increase in basal oxygen consumption (2).

In 1928, two investigators (3), working with dogs, noted a marked elevation in basal metabolism after subcutaneous injection of 15 to 30 mgm. of histamine.

Also, in 1929, it was found that after subcutaneous injection of 0.4 to 0.7 mgm. of histamine phosphate into a trained person, a small elevation of metabolism was observed, on an average of 7 per cent (4).

In 1934, two workers (5) gave 1 mgm. of histamine subcutaneously to each of 17 human subjects. Eleven showed a considerable decrease in basal metabolic rate, while 6 were within limits of error. They explained their work on the basis of stimulation of the parasympathetic nervous system and paralysis of the sympathetic system. It should be noted that the metabolic measurements were made some time after the injection.

In 1932, the effect of histamine administered intravenously on the basal metabolism of human

subjects was reported (6). An elevation in basal metabolism was obtained as high as 50 per cent in some cases. Considerable variation was shown in degree of elevation of metabolism with similar rates of injection. As far as we know, these investigators were the first to determine the effect of histamine on basal metabolism using the drug intravenously.

During clinical investigations at the Mayo Clinic, the occasion arose to investigate the effect on metabolism of histamine administered intravenously. The results of our studies are consistent with the observations of the latter group of workers (6).

PROCEDURE

Two sets of experiments were performed. In both sets, the basal metabolic rate was determined before the administration of histamine but in Group I the metabolic rate was not determined again until after the completion of the injection; while in Group II, the metabolic rate was determined during the injection.

Group I. Twenty-three subjects were studied by the Tissot method. Thirteen were men and ranged in age from 22 to 60 years, the average age being 50 years. Ten were women and ranged in age from 26 to 64 years, with an average age of 44 years. Such diagnoses as internal squint, allergic arthritis, headache, angioneurotic edema, postural vertigo, arteriovenous fistula, tinnitus, Parkinson's disease, Ménière's disease, chronic nervous exhaustion, torticollis, chronic infectious arthritis, multiple sclerosis and cyclic edema had been made. The metabolic rate was determined before and after the intravenous administration of histamine. All subjects studied had been without food for 15 or more hours. Of the 23 subjects, 16 showed an increase in metabolic rate of from +1 per cent to +25 per cent, 5 showed a decrease of from -1 per cent to -6 per cent and 2 did not show any change. It appeared likely that a considerable amount of the effect of histamine was being missed by waiting until the completion of the injection. Therefore, a second group of subjects was studied by a different procedure.

Group II. Eleven subjects were studied by means of a modified Benedict-Roth apparatus. Five of these were women and 6 were men. The former ranged in age from

¹ Since this paper was written Dr. Peters has entered the armed forces and is now First Lieutenant, Medical Corps, Army of the United States.

18 to 34 years, with an average of 27 years, and the latter ranged in age from 31 to 59 years, with an average of 38 years. The metabolic rate of these subjects was determined before, in the course of, and after the intravenous injection of, histamine. They had such diagnoses as Ménière's disease, headache, chronic nervous exhaustion, purpura or urticaria, or were normal subjects. The procedure for this group was to allow the fasting subject to rest quietly in bed for 20 to 30 minutes. Then the basal rate was determined. The oxygen mask was then removed, and the subject was allowed to rest for 10 minutes. Then, after a subcutaneous injection of 1 per cent solution of procaine hydrochloride over an antecubital vein, an intravenous injection of physiologic saline solution at rates varying from 60 to 240 drops per minute was begun, and the metabolic rate was determined again. After this, a 1:250,000 solution of histamine diphosphate was substituted for the saline solution, and rates of injection, usually 4 varying from 0.0066 to 0.0528 mgm. of histamine base per minute, were used. During and after each increment in rate of injection, a determination of oxygen consumption was made. A rest period of 10 minutes with the mask off was allowed between changes of injection rate and before a final basal metabolic rate was determined at the conclusion of the experiment.

As a comparative study, a 1:1,000,000 dilution of epinephrine and physiologic saline solution alone were injected intravenously in a similar fashion as the histamine solution, and the effect on the metabolic rate was observed.

Five subjects were studied under absolutely controlled environmental conditions. The subjects were in a temperature of 78° F. and a relative humidity of 40 per cent. The oxygen content of the blood from the antecubital

vein was determined before and during the height of the administration of histamine.

RESULTS

The results of this study are summarized in Tables I to IV. In Table I, it is easily observed that increasing amounts of histamine administered intravenously produced a corresponding rise in consumption of oxygen and in the ventilation rate. Additional control observations with saline solution were made on the 11 subjects of Group II, showing that no increase in metabolic rate took place with saline solution, though consistent increase was produced by histamine given during subsequent periods to the same subjects (Table I). Also histamine produced a rise in cardiac rate and a lowering of the blood pressure. The effect on the metabolic rate, however, was soon dissipated after discontinuance of administration of the drug. It is interesting to note that with each doubling of the dose of histamine, the metabolic rate increased approximately twice its former value. At 30 drops per minute, the basal metabolic rate was 4 per cent, at 60 drops it was 14 per cent, at 120 drops it was 29 per cent, and at 240 drops it was 58 per cent.

Table II shows the results obtained with a 1:1,000,000 solution of epinephrine. This solu-

TABLE I
Effect of histamine on basal metabolism (mean results in eleven cases)

Procedure	Oxygen consumption		Ventilation rate	Pulse rate	Blood pressure		Increase in B.M.R.
	total ml. per minute	ml. per square meter per minute			Systolic	Diastolic	
Basal	236.5	126.2	6.36	66	114	73	per cent
Saline solution, 60 to 240 drops per minute	229.0	123.4	6.18	62	114	75	
Histamine 30 drops (0.006 mgm.) per minute	243.0	129.7	6.78	69	108	71	4
60 drops (0.012 mgm.) per minute	266.0	142.2	7.16	82	108	67	14
120 drops (0.024 mgm.) per minute	300.2	160.0	8.02	98	100	60	29
240 drops (0.048 mgm.) per minute	367.8	198.0	8.92	116	93	51	58
Ten minutes after histamine	234.8	125.5	6.21	61	116	77	

TABLE II
Effect of epinephrine (1:1,000,000) on basal metabolism (1 case)

Procedure	Oxygen consumption		Ventilation rate	Pulse rate	Blood pressure		Increase in B.M.R.
					Systolic	Diastolic	
Basal	<i>total ml. per minute</i> 204.4	<i>ml. per square meter per minute</i> 112	<i>liters per minute</i> 5.11	56	104	72	<i>per cent</i>
Saline solution, 240 drops per minute	193.6	106	4.93	56	106	74	
Epinephrine 30 drops per minute	200.8	110	5.20	66	103	68	0
60 drops per minute	208.0	114	5.92	68	116	76	4
120 drops per minute	222.3	121	7.00	74	118	74	12
240 drops per minute	222.3	121	8.07	84	156	94	12
Ten minutes after epinephrine	211.6	116	5.92	64	116	78	

TABLE III
Effect of physiologic saline solution on basal metabolism (1 case)

Procedure	Oxygen consumption		Ventilation rate	Pulse rate	Blood pressure	
					Systolic	Diastolic
Basal	<i>total ml. per minute</i> 201.3	<i>ml. per square meter per minute</i> 110.0	<i>liters per minute</i> 5.04	50	106	72
Saline solution 7 drops per minute	204.9	112.0	5.13	52	104	74
30 drops per minute	169.6	92.7	4.95	50	114	74
60 drops per minute	190.3	104.0	5.30	50	114	74
120 drops per minute	183.0	100.0	5.30	50	110	78
240 drops per minute	183.0	100.0	5.48	48	112	78
Ten minutes after saline solution	208.6	114.0	3.80	48	112	80

TABLE IV
Increase in oxygen content of venous blood during histamine administration*

Case	Histamine	Before histamine		After histamine		Capacity	Increase in saturation
		Oxygen content	Saturation	Oxygen content	Saturation		
1	<i>drops per minute</i> 120	<i>volumes per cent</i> 11.4	<i>per cent</i> 57	<i>volumes per cent</i> 16.7	<i>per cent</i> 84	<i>volumes per cent</i> 20.0	<i>per cent</i> 27
2	160	11.5	60	18.2	94.3	19.3	34
3	120	14.1	69	16.0	79	20.3	10
4	20 to 30	13.1	62	16.9	74	22.5	12
5	20 to 30	13.1	66	18.0	88	20.4	22
Average							21

* The subjects were in a basal state, that is without food, and in an environmental temperature of 72° F. and 60 per cent humidity.

† Histamine solution 1:100,000.

tion produced only a slight rise in oxygen consumption or an elevation of the basal metabolic rate of 12 per cent. The ventilation rate, cardiac rate, and blood pressure, however, were considerably elevated. The effect of the epinephrine seemed to be more prolonged than that of the histamine after the intravenous injection was discontinued.

Table III shows the effect of physiologic saline solution given in the same way as the solutions of histamine and of epinephrine. The saline solution

did not produce any increase of oxygen consumption.

As shown in Table IV, there was an average increase in oxygen saturation of the blood from the antecubital veins of 21 per cent. The percentage of increase ranged from 10 to 34. The blood in each case was withdrawn under oil without the use of a tourniquet, and the determinations of oxygen were made by the Van Slyke gasometric method. Each subject had been lying in a horizontal position, at rest, for at least 40 minutes,

ACCUMULATIVE EFFECT OF HISTAMINE ON OXYGEN CONSUMPTION

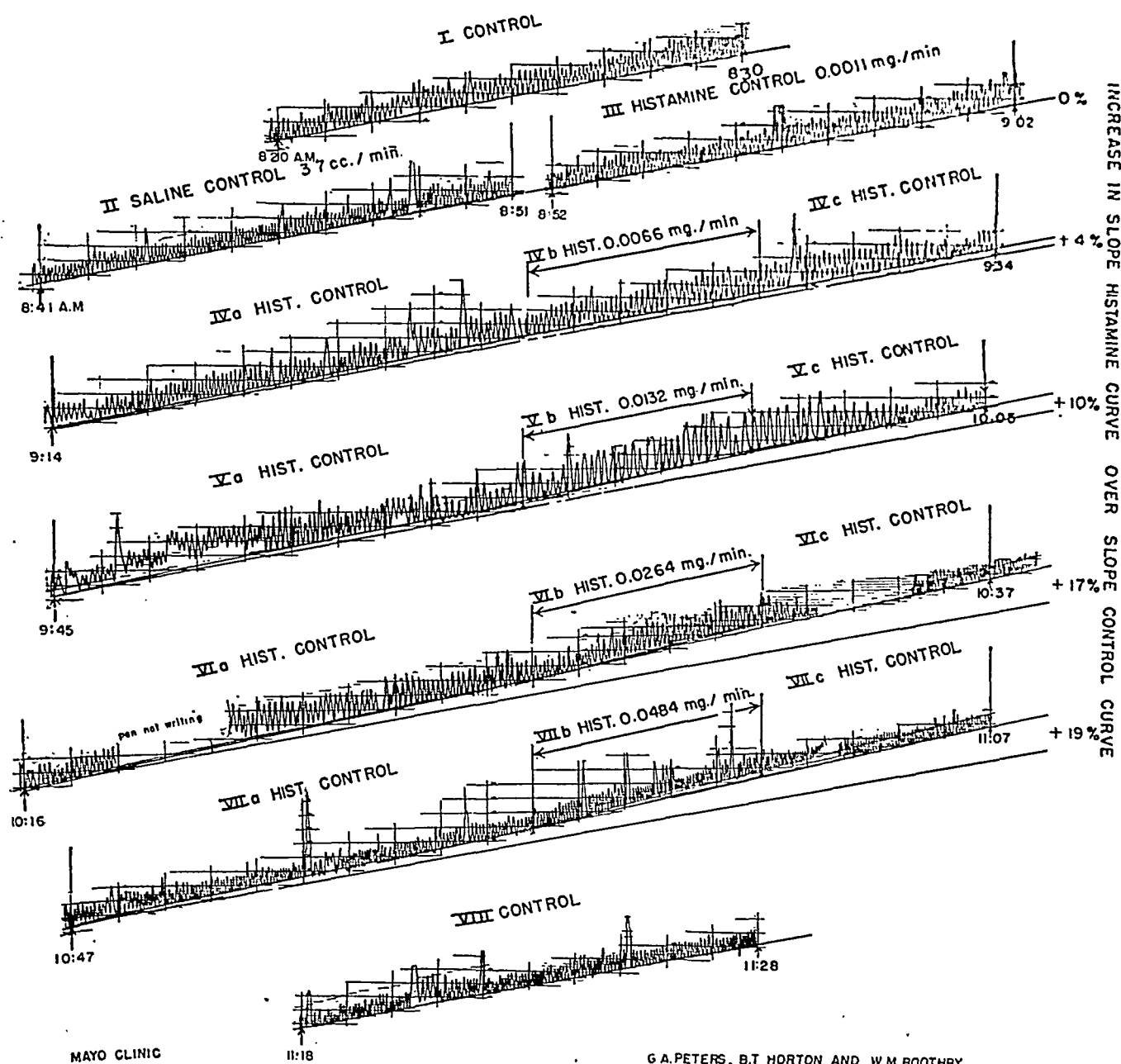


FIG. 1. METABOLIC KYMOGRAPHIC RECORD OF 1 OF THE SUBJECTS, SHOWING THE EFFECT OF HISTAMINE ON METABOLISM

before the specimens of blood were withdrawn. The gross appearance of the blood in the veins was observed to be redder and more arterial at the height of the injection of histamine than before injection.

Figure 1 reproduces an actual record of a normal subject, aged 26 years, taken from the Benedict-Roth machine. The kymographic tracing shows the increase in the metabolic rate of from 0 per cent to +19 per cent. It illustrates well the effect on the respiratory rate and how quickly the effect of histamine on oxygen consumption is produced and lost. One subject, a man, aged 59 years, showed a rise in metabolic rate of from -14 per cent to +96 per cent. These 2 subjects represent the extremes in low and high response to histamine respectively. The other subjects were intermediate in their response to histamine. With the same rates of injection, considerable variation of the degree of elevated metabolism was shown by the subjects.

COMMENT

The basal metabolic rate is a measure of the oxygen consumption for each square meter of body surface; it has been shown to be a measure of heat production. Any substance which brings about an increase of peripheral circulation might cause a greater loss of heat from the body and thus elevate the metabolic rate.

In man, histamine dilates the arterioles and capillaries, and it also causes an increase in capillary permeability. In general, it acts as a secretagogue, particularly to the gastric mucosa. Both actions, no doubt, require the expenditure of energy and may play a part in the increased oxygen consumption. The peripheral vasodilatation resulting from the intravenous administration of histamine is not uniform throughout the human body. Because of the vasomotor gradient, as it exists in man, vasodilatation occurs promptly in the face and neck in most subjects. When the histamine is administered at increased rates, a gradually increasing vasodilatation spreads over the thorax, abdomen, and upper extremities, and it may even extend to the lower extremities. This vasodilatation is inclined to be splotchy in its distribution, especially over the thorax, abdomen, and upper extremities. This is more marked in blonds and redheads than in brunets.

We realize that kymographic records obtained with the closed circuit metabolism apparatus (Benedict-Roth) are subject to errors, and it is possible to explain the apparent increase in oxygen consumption on the basis of alterations in the tidal air. For example, as the functional residual air is gradually expelled during increased respiration, the graph would naturally indicate an increase in oxygen consumption, whereas no actual increase had taken place. To rule out such errors, the same subject, in several instances, was studied for 3 or more hours on 3 to 4 successive days, and no alterations in tidal air could be demonstrated as a possible explanation for increased oxygen consumption. Furthermore, such an explanation could not account for the increased oxygen consumption, as measured by the Tissot method.

CONCLUSIONS

Histamine has been shown to increase oxygen consumption during intravenous injection, and the increase as measured is roughly proportional to the rate at which histamine is given in one series. This effect ceases soon after administration is stopped, thus differing from the effect of such "calorigenic" substances as thyroxine. Further study is needed to determine the reason for this increased oxygen consumption.

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LIVER FUNCTION TESTS IN CHRONIC RELAPSING VIVAX MALARIA¹

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Hepatic function in chronic relapsing malaria caused by *Plasmodium vivax* and treated principally by atabrine has been investigated at Harmon General Hospital in 317 soldiers. This study seemed advisable because of previous reports that impairment of liver function may occur in both naturally-acquired (1 to 3) and induced malaria (4). Several types of tests were performed on our patients both during recurrent attacks and at various intervals afterwards. Most of these same tests were also performed on healthy individuals to determine whether in our laboratory the methods gave normal ranges of values corresponding to those found in the literature. This made it possible to compare the findings in our patients with those of our healthy control series, as well as with the results of other investigators.

The liver function tests used may be grouped roughly into 3 categories: (a) those in which hepatic dysfunction is suspected when abnormal values are found—bromsulfalein, cephalin flocculation, galactose tolerance and hippuric acid tests; (b) those in which an abnormal value may be associated with increased red blood cell destruction and/or hepatic dysfunction—icterus index, serum bilirubin concentration, and urine urobilinogen concentration; and (c) quantitative determinations of some constituents of blood serum occasionally found to yield abnormal results in persons with hepatic disease—albumin, globulin, total protein, cholesterol and phosphatase.

METHODS

1. *Bromsulfalein* (5): The amount of dye injected intravenously was 5 mgm. per kgm. of body weight. The permanent standards used were supplied by Hynson, Westcott and Dunning. Since the standards were prepared for the 2 mgm. test, the calculations were made by dividing the readings obtained by 2.5.

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2. *Cephalin flocculation test* (6, 7): The cephalin-cholesterol antigens were obtained from the Difco Company (Digestive Ferments Company) of Detroit, Michigan, and from the Wilson Laboratories of Chicago, Illinois. The test was performed according to Hanger's method with the exception that most of the tests were kept in the dark since recently light has been reported as affecting the results (8).

3. *Galactose tolerance test*: Forty grams of galactose were given by mouth to fasting subjects. The amount of sugar excreted in the urine during the next 5 hours was determined by Benedict's quantitative method using a solution which had been standardized against galactose. The results were reported as galactose.

4. *Intravenous hippuric acid test* (9): Sodium benzoate (1.77 grams) was injected intravenously, and the urinary excretion of hippuric acid for a period of 1 hour was determined. The hippuric acid was salted out of the acidified urine with ammonium sulfate (0.5 gram per ml.) at 20° C. for 1 hour. The precipitate was filtered with suction and was washed with three 5 ml. portions of ice-cold water. In the calculation of the results, correction for the solubility of hippuric acid was made by adding 0.1 gram per 100 ml. of urine to the weight of precipitate obtained.

5. *Icterus index*: The color of the serum was compared with those of standard potassium dichromate solutions in 75 × 12 mm. test tubes. The serum was then diluted 1:2 and 1:3 with normal physiological saline, and the colors of these dilutions were matched with the standards as checks on the original value obtained. With an occasional serum, poor checks were obtained with the 2 dilutions, and in such a case the serum was diluted further until consistent values were obtained.

6. *Serum bilirubin*: The method used was the Thannhauser-Andersen modification (10) of the Van den Bergh test. The permanent cobalt sulfate standards of McNee and Keefer were used (11).

7. *Urine urobilinogen*: A modification of the Wallace and Diamond (12) serial dilution method was used on fresh morning specimens. The reagent was prepared by dissolving 0.15 gram paradimethylaminobenzaldehyde in 100 ml. of 20 per cent hydrochloric acid. Tests were routinely performed on a 1:5 dilution. Urine specimens yielding positive tests in this dilution were diluted further and tested again. The highest dilution in which a positive test was obtained was recorded.

8. *Serum albumin and globulin*: The method of Minot and Keller (13) was used.

9. *Serum cholesterol*: The method of Forbes (14) was used.

10. *Serum phosphatase*: The method of Bodansky (15) was used. The final phosphorus determinations were made by the method of Fiske and Subbarow (16).

RESULTS

Bromsulfalein test: Control tests were run on 100 normal healthy subjects (Table I). At 45 minutes, 44 per cent of the individuals showed no retention of dyestuff, while 55 per cent showed

TABLE I

Results of bromsulfalein tests at 45 minutes using 5 mgm. per kilogram body weight

Dye retention	100 "Normal" controls	49 Patients on second or third day of attack	97 Patients on sixth or seventh day of attack	149 Men following recurrent attacks
per cent	per cent	per cent	per cent	per cent
0	44	53	69	60
2	41	27	29	24
4	14	16	1	13
6	1			
8		2		3
10			1	
12		2		

2 or 4 per cent retention. One man, who was presumably a healthy individual, had 6 per cent retention. It was reported recently (17) that with 5 mgm. of dye, there was no retention at 45 minutes, while others (5) accept up to 6 per cent retention at 60 minutes as normal. It was concluded that, under our conditions of testing, as much as 4 per cent retention at 45 minutes was to be considered normal.

During 49 recurrent attacks of malaria, the bromsulfalein test was done on the second or third day of the attack and repeated 3 or 4 days later. On the second and third days, 96 per cent of the results fell within the normal range. One man had 8 per cent retention and another had 12 per cent retention, but 3 or 4 days later both were normal. Ninety-seven patients (including the 49 who had been tested on the second or third day) were studied on the sixth or seventh day of an attack; the results were all normal at 45 minutes with the exception of that of 1 man in whom there was 10 per cent retention. Tests were performed on 149 men at intervals of from 2 to 44 weeks after recurrent attacks of malaria. Of these men, 45 had had normal findings when previously tested from the second to the seventh days from the start of an attack and again showed normal results

following attacks. Of the remaining 104 men, there were 4 who had 8 per cent retention at 45 minutes. Therefore, with reference to our control figures, only 4 per cent of the patients had abnormal values on the second or third days of an attack, 1 per cent on the sixth or seventh days of an attack, and 3 per cent after an attack. The total number of abnormal results by the bromsulfalein test, either during or after an attack was 7, or 2.4 per cent of 295 tests.

Cephalin flocculation test: Duplicate tests were performed with each serum using the Difco and Wilson antigens. Sera were obtained on the first 5 days during 42 recurrent attacks of malaria (Table II). With the Difco antigen, 82 per cent of the tests were positive, and each of the 42 patients had at least 1 positive test during the 5-day period. With the Wilson antigen, 21 per cent of the tests were positive, and in only 50 per cent of the 42 patients was a positive test obtained during the 5 days.

TABLE II

Cephalin flocculation tests for five consecutive days during 42 recurrent attacks of malaria (comparing Difco and Wilson antigens on same sera)

Difco antigen

Day	0	1+	2+	3+	4+
First	12	3	7	14	6
Second	6	4	8	10	14
Third	5	5	5	13	14
Fourth	8	6	8	8	12
Fifth	6	6	6	12	12
Total	37	24	34	57	58

Wilson antigen

Day	0	1+	2+	3+	4+
First	38	1	0	2	1
Second	30	4	0	7	1
Third	33	2	1	5	1
Fourth	35	1	1	2	3
Fifth	30	1	3	5	3
Total	166	9	5	21	9

In duplicate tests on the sera of 204 men, 2 to 44 weeks following recurrent attacks of malaria, Table III shows that with the Difco antigen 20 per cent of the tests were positive, and with the Wilson antigen 5 per cent were positive. Each of the latter sera also gave positive results with the Difco antigen. Of the 41 men giving the positive tests with the Difco antigen, 24 were available for

another test 5 weeks later without an intervening attack of malaria. At that time, tests of 10 of the sera were still positive. This group of 24 men included the 10 men who had given positive tests with the Wilson antigen and on re-testing 5 weeks later, without an intervening attack of malaria, only 2 remained positive.

TABLE III

Results of cephalin flocculation tests in 204 patients following recurrent attacks of malaria

Flocculation	0	1+	2+	3+	4+
Difco antigen	163	4	8	16	13
Wilson antigen	194	0	0	7	3

These results with duplicate tests, using the Difco and Wilson antigens, show that there is a substantial difference in the sensitivity of the two antigens. There is also a marked difference in results between the number of positive tests during the attacks and following the attacks when the results with the same antigen are compared. In spite of the difference in sensitivity between the 2 antigens, it is apparent that the disturbance producing the greater number of positive tests during attacks is of a transient character.

Galactose tolerance test: The galactose tolerance test was performed on 207 men from 4 to 52 weeks after an attack of malaria (Table IV). Only 7, or 3 per cent, of the men excreted over the accepted normal amount of 3 grams of galactose in 5 hours. The average excretion for the entire group at hourly intervals was as follows: first hour, 0.80 gram; second hour, 0.35 gram; third hour, 0.08 gram; fourth hour, 0.05 gram; and fifth hour, 0.02 gram.

TABLE IV

Galactose tolerance tests in 207 men following recurrent attacks of malaria

Number in group	Grams of galactose excreted in five hours
18	0.0
77	0.1-0.9
42	1.0-1.5
34	1.51-2.0
29	2.01-3.0
5	3.01-4.0
2	4.01-5.0

Intravenous hippuric acid test: The intravenous hippuric acid test using 1.77 grams of sodium benzoate was performed on 26 normal individuals and on 56 men following recurrent attacks of

malaria (Table V). According to Quick (9), 1 gram or more of hippuric acid is excreted in 1 hour by a normal person. Certain workers (18) have pointed out that the amount of hippuric acid excreted bears a relationship to body weight. In their series of 46 normal individuals, they obtained values as low as 0.65 gram, and 9 per cent of their normal individuals excreted less than 0.90 gram. In our smaller series of 26 normals, 11 per cent excreted less than 0.90 gram. In our 56 men following attacks of malaria, 23 per cent excreted less than 0.90 gram.

TABLE V

Intravenous hippuric acid test (reported as hippuric acid)

26 Normal healthy controls			56 Men following attacks of malaria		
Number	Grams hippuric acid	Per cent of group	Number	Grams hippuric acid	Per cent of group
2	0.33-0.59	7.7	2	0.33-0.59	3.6
1	0.60-0.79	3.9	5	0.60-0.79	8.9
0	0.80-0.89	0.0	6	0.80-0.89	10.7
3	0.90-0.99	11.5	9	0.90-0.99	16.1
5	1.00-1.09	19.3	10	1.00-1.09	17.9
6	1.10-1.19	23.0	13	1.10-1.19	23.2
6	1.20-1.29	23.0	5	1.20-1.29	8.9
3	1.30-1.58	11.5	6	1.30-1.58	10.7

Icterus index and serum bilirubin concentration: It is customary to determine the icterus index and the bilirubin concentration of blood serum on fasting specimens. In our studies, most of the patients were having many specimens of blood taken for plasma atabrine determinations 2 to 4 hours after breakfast. In order to minimize the number of venepunctures, sera from these same specimens were used for the icterus index and bilirubin determinations. In doing this, it seemed essential that control values from non-fasting sera of healthy soldiers should be compared with the results in our patients, and in addition that the non-fasting specimens from healthy soldiers should be compared with fasting specimens to determine whether the results might be different. Sera were obtained from 562 normal healthy soldiers 2 to 4 hours after breakfast and, in addition, fasting sera from 115 of these men were obtained on the same day. Seven per cent of the fasting values for the icterus index were greater than 8 units, and 9 per cent of the fasting values for the serum bilirubin were greater than 0.50 mgm. per 100 ml. The results obtained with postprandial

TABLE VI

Icterus index and serum bilirubin on the same postprandial sera of 562 normal healthy soldiers

Icterus index				Serum bilirubin			
Units	Number in group	Per cent	Cumulative per cent	Mgm. per 100 ml.	Number in group	Per cent	Cumulative per cent
2	2	0.4	0.4	<0.10	183	32.6	32.6
3	75	13.4	13.8	0.10	116	20.7	53.3
4	114	20.3	34.1	0.15	86	15.3	68.6
5	123	21.9	56.0	0.20	66	11.7	80.3
6	115	20.5	76.5	0.25	31	5.5	85.8
7	75	13.4	89.9	0.30	12	2.1	87.9
8	26	4.6	94.5	0.35	14	2.5	90.4
9	13	2.3	96.8	0.40	18	3.2	93.6
10	5	0.9	97.7	0.45	9	1.6	95.2
11	6	1.1	98.8	0.50	8	1.4	96.6
12	3	0.5	99.3	0.55	5	0.9	97.5
13	3	0.5	99.8	0.60	4	0.7	98.2
14	1	0.2	99.9	0.65	1	0.2	98.4
				0.80	5	0.9	99.3
				0.85	1	0.2	99.5
				0.90	1	0.2	99.7
				1.00	1	0.2	99.9
				1.50	1	0.2	100.1
Total 562				Total 562			

specimens tended to be slightly lower (Table VI). Five per cent of these values for the icterus index were greater than 8 units and 3 per cent of the values for serum bilirubin were greater than 0.50 mgm. per 100 ml.

During 108 attacks of malaria, both the icterus index and serum bilirubin concentration were determined on 5 successive days from the beginning of the attack. Following 118 attacks, the same tests were performed at intervals of 1, 2 and 3 weeks after the attack. From the second to the fifteenth week after attacks, these same tests were performed on 997 specimens from 260 men. The results are shown in Figure 1. On the first day of the attack, 16 per cent of the values for the icterus index were greater than 8 units and 31 per cent greater than 7 units. By the fourth day, the percentage over 8 units had dropped to 1 per cent, a value smaller than the 5 per cent found in normal healthy soldiers. Similarly, the percentage greater than 7 units dropped to 3 per cent, whereas it was 10 per cent in the normal

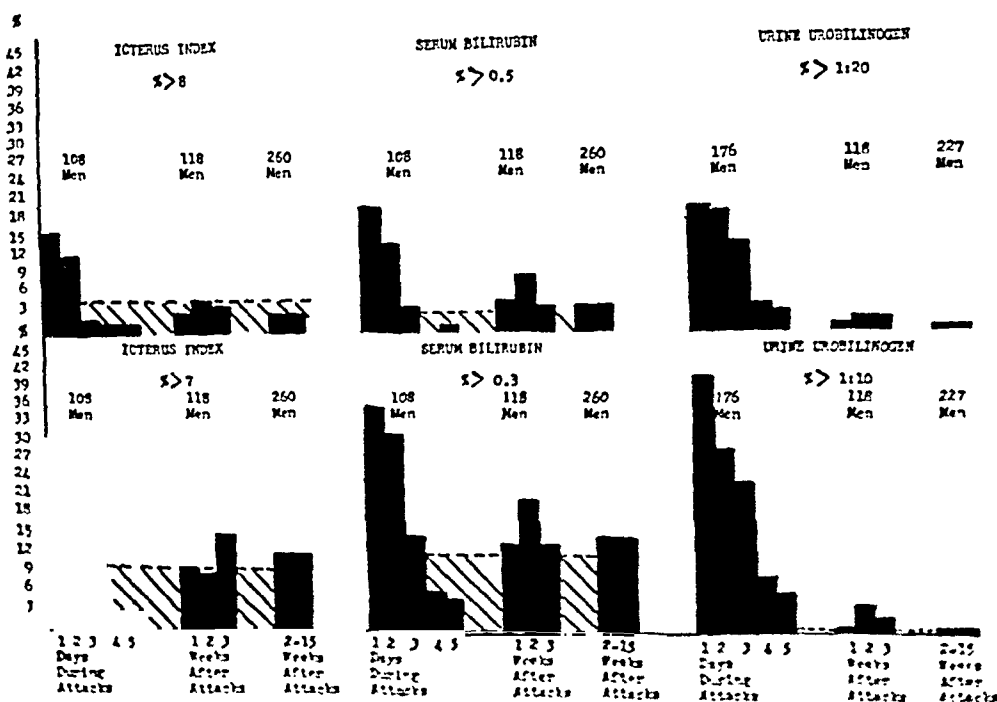


FIG. 1. ICTERUS INDEX, SERUM BILIRUBIN, AND URINE UROBILINOGEN DURING AND AFTER RECURRENT ATTACKS OF MALARIA

(Compared with normal healthy men)

Keys: Solid blocks—patients with malaria 6577 tests

Hatching—normal healthy controls 1542 tests

Total tests 8119

healthy soldiers. From 2 to 15 weeks after the attacks, the percentages greater than 8 and 7 units respectively were essentially the same as those encountered in the control group. Similar findings were observed with the serum bilirubin concentrations during the same periods. The increase in number of elevated values during the first part of the attack with subsequent fall to below normal and later elevation to about the normal level occurred with both tests. However, the degree of elevation was not great nor did it occur in every single individual.

Urine urobilinogen test: The test for urobilinogen was performed on fresh morning urine specimens of 960 individuals without malaria (Table VII). Of these, 418 were normal healthy soldiers. Only 3 per cent gave positive results in a maximum dilution of 1:10 and 1 per cent in a maximum dilution of 1:20. The remainder

TABLE VII

Urine urobilinogen on 960 individuals without malaria

Normal healthy men		
Dilution	Number	Per cent
<1:5	281	67
1:5	122	29
1:10	11	3
1:20	4	1
Total	418	100
General hospital patients		
Dilution	Number	Per cent
<1:5	324	73
1:5	108	25
1:10	7	2
1:20	2	<1
1:50	1	<1
Total	442	100

of the group were 442 general hospital patients with no apparent liver damage. Only 2 per cent gave positive results at a dilution of 1:10. At a 1:20 dilution, 0.5 per cent were positive and a single individual with pneumonia was positive in a dilution of 1:50. These figures tend to show that in healthy men or in patients without apparent liver damage or obvious hemolysis, values above 1:10 are uncommon.

During 176 attacks of malaria, urine urobilinogen tests were performed on 5 successive days from the beginning of the attack. Following 118 attacks, the same test was performed at intervals of 1, 2 and 3 weeks. From the second to the fifteenth week, the same test was performed on an additional 1561 specimens from 227 men. The

results are shown in Figure 1. On the first day of the attack, 20 per cent of the tests were positive in a dilution greater than 1:20, and 41 per cent were positive in a dilution greater than 1:10. The number of elevated concentrations steadily decreased so that by the fifth day but few were above the normal limit. From 2 to 15 weeks after attacks the values were practically identical with those obtained with the normal healthy controls.

Serum albumin, globulin and total protein: The serum albumin, globulin and total protein were determined from 1 to 26 weeks following recurrent attacks of malaria in 172 men. All values for serum albumin were within normal limits. In 3 men values above 2.7 grams per 100 ml. were obtained for the globulin, with the highest being 3.1. In only 2 men were the values for total protein below 6.0 grams per 100 ml., with the values being 5.0 and 5.8 respectively. It is evident that hyperglobulinemia and hypoproteinemia were not frequent in this group of patients.

Cholesterol: Cholesterol determinations were made on 156 fasting patients after recurrent attacks of malaria. None had a value below 150 mgm. per 100 ml. Thirteen men had values between 251 and 260, 5 between 261 and 270, 3 between 271 and 280 and 1 between 281 and 290 mgm. per 100 ml. This gave a total of 22 men with values above 250 mgm.

Phosphatase: The serum phosphatase was determined in 191 men following recurrent attacks of malaria. All values were below 5 Bodansky units.

DISCUSSION

Liver function in naturally-acquired malaria (1 to 3) has been previously studied in several countries where the disease is endemic. It has also been studied in induced malaria (4) used in the treatment of neurosyphilis. Frequently only a single test has been employed; when several tests have been used, the group studied has been quite small. Urobilinogenuria, hyperbilirubinemia, decrease in serum protein and impairment of glyco-genetic function, as indicated by the galactose tolerance test, have been observed in naturally-acquired malaria (1 to 4). Transient disturbances in liver function have been reported in induced malaria on a basis of moderate bromsulfalein retention, diminished hippuric acid excretion and a

strongly-positive cephalin flocculation (4). The impairment was said by these investigators to disappear within 3 to 6 weeks after termination of the malaria.

The patients at Harmon General Hospital formed a desirable group upon which to perform liver function tests. The group was homogeneous with respect to age period, color, endemic area in which the *Plasmodium vivax* infection was acquired, and chronicity of disease as indicated by an average of 9 recurrent attacks in about 17 months. Clinically, these patients frequently complained of vague gastro-intestinal symptoms and were easily fatigued, even after months of convalescent care. During 308 observed attacks in these and some other patients, not included in this particular study, 32 per cent had pain in the right side, chiefly in the upper quadrant, and 12 per cent had palpable livers. All had had prolonged suppressive treatment with atabrine while overseas, as well as thorough treatment with it during recurrent attacks.

The majority of these patients were available for study for a period of 6 months. This created an opportunity to perform a large series of tests both during and after recurrent attacks of malaria and to observe whether there were any transient or possibly permanent abnormalities in liver function. During the acute attacks, with those tests which gave presumptive evidence of liver dysfunction, less than 5 per cent of the bromsulfalein tests were abnormal. With the cephalin flocculation test 82 per cent of the tests were positive with the Difco antigen and 21 per cent with the Wilson antigen. One worker (19) has recently presented evidence that either qualitative or quantitative changes in the fractions of serum protein may be responsible for cephalin flocculation. With those tests which are associated with increased hemolysis and/or disturbance in liver function; namely, the icterus index, serum bilirubin concentration and urine urobilinogen concentration, there were frequent abnormal values during the first part of the attacks with return to normal by the fifth day.

Following attacks of malaria, there was positive cephalin flocculation in 20 per cent of the tests with the Difco antigen and 5 per cent with the Wilson antigen. The results with the Wilson antigen are more in accord with the findings of 3

per cent abnormal values in both the bromsulfalein and galactose tolerance tests than are those of the Difco antigen. With the intravenous hippuric acid test, 11 per cent of the values were abnormal when compared to the control series. Evaluation of the group of tests as a whole shows that although there may be some transient disturbance during the recurrent attacks, there is at present little evidence of permanent hepatic dysfunction as a result of chronic relapsing vivax malaria treated principally by atabrine.

SUMMARY

The results obtained in the present study may be summarized as follows:

1. The results with the bromsulfalein test showed that:

- (a) On the second or third days of an attack in 49 men, 4 per cent were abnormal.

- (b) On the sixth or seventh days of an attack in 97 men, 1 per cent were abnormal.

- (c) Following recurrent attacks in 149 men, 3 per cent were abnormal.

2. The percentages of positive cephalin flocculation tests during and after attacks of malaria with 2 different antigens were as follows:

- (a) Of tests performed on the first 5 days during 42 recurrent attacks, 82 per cent were positive with the Difco antigen, and 21 per cent were positive with the Wilson antigen.

- (b) Following 204 attacks of malaria, 20 per cent of these tests were positive with the Difco antigen, and 5 per cent with the Wilson antigen.

3. Galactose tolerance tests were performed on 207 men following recurrent attacks of malaria; of these 3 per cent were abnormal.

4. The intravenous hippuric acid test was performed on 56 patients following attacks of malaria. Twenty-three per cent of this group excreted less than 0.90 gram of hippuric acid in 1 hour.

5. The icterus index and serum bilirubin concentration tests were determined on the same sera both during and after attacks of malaria.

- (a) At the onset of 118 attacks, 16 per cent of the values for the icterus index were greater than 8 units, and 20 per cent of those for the bilirubin concentration were greater than 0.50 mgm. per 100 ml. of serum. By the fifth day, and for the following 15 weeks, the values were approximately normal.

(b) The degree of elevation above normal in both tests was not great, nor did elevation occur in every individual.

6. The urine urobilinogen test was performed both during and after attacks of malaria. On the first day of 176 attacks, 20 per cent of the tests were positive at a dilution greater than 1:20. By the fifth day but few tests showed values above normal although an occasional individual had an elevated figure for a short period of time with the rest being about normal within 2 to 15 weeks after an attack.

7. In 172 men following recurrent attacks of malaria, the albumin, globulin and total protein were normal in all but 5 men.

8. In 156 men following recurrent attacks of malaria, the serum cholesterol was essentially normal.

9. In 191 men following recurrent attacks of malaria, the phosphatase was normal.

CONCLUSION

Liver function tests in 317 patients with chronic relapsing vivax malaria treated principally by atabrine disclosed evidence of some transient disturbance during recurrent attacks but gave little or no indication of permanent hepatic dysfunction.

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THE LIPIDS OF SERUM AND LIVER IN PATIENTS WITH HEPATIC DISEASES¹

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The investigations of dietary fatty livers have thrown into prominent relief the function of the liver in the intermediary metabolism of lipids. The discovery that these disorders may be remedied or prevented by lipotropic agents may have important therapeutic implications since there is some evidence that the chemical and anatomical disturbances of the liver produced by some diseases and poisons resemble those found in dietary livers and that they may be amenable to the action of these lipotropic agents (1 to 3). These were among the reasons that led to an examination of the serum lipids in patients with diseases of the liver and pancreas and obstruction of the biliary system. Such studies have been made before but have usually been confined to the determination of cholesterol, with or without fractionation, often by methods of doubtful accuracy. Because the interplay of the various lipid components appears to have significance apart from the concentration of any single constituent, as often as possible the lipids were fractionated into their 4 major components: fatty acids, lipid phosphorus, total cholesterol, and free cholesterol. From the values thus obtained, the cholesterol esters and neutral fat were estimated (4). In addition, samples of livers, secured at autopsy from some of the patients who had been studied, were analyzed for the same compounds. Finally, the effects of lipotropic agents on the course of the disease in certain of the patients are mentioned.

EXPERIMENTAL

Clinical material. Altogether 174 determinations of serum lipids were made on 70 patients. On some occasions, especially in the earlier studies, the lipids were not completely fractionated. The numbers of analyses for the various lipid frac-

tions, therefore, vary and complete comparisons cannot be made in all instances. All fractions were, however, measured in 125 observations on 54 of the cases. Proteins and protein fractions also were determined on 146 occasions.

The cases can be roughly classified under the following headings:

Diagnosis	Number of cases	
Obstruction of the common bile duct	15	
Infectious hepatitis	11	
Toxic hepatitis	6	
Certain		3
Questionable		3
Portal cirrhosis	28	
Typical		16
Atypical		12
Biliary cirrhosis	8	
Typical		6
Atypical		2
Acute hemorrhagic pancreatitis	2	

Some of these categories require explanation. Obstruction of the common bile duct includes patients with common duct stones, carcinoma of the pancreas, and other tumors which occluded the duct. In most instances, the diagnosis was confirmed by operation or post mortem examination. In all, there was evidence of acholia at some time. Studies were made before and after relief of obstruction in many cases; in a few only after operation; in others, who had inoperable conditions, at intervals during their illness.

The group termed infectious hepatitis is somewhat heterogeneous. All had febrile illnesses associated with icterus, usually accompanied by enlargement and tenderness of the liver. No evidence of exposure to hepatic poisons could be elicited from any of them. One had an antecedent history suggesting cholelithiasis of the gall-bladder, and x-rays at that time had revealed imperfect filling of the gall-bladder. In 4 instances, the liver remained somewhat enlarged after the acute attacks had subsided. In 1 of

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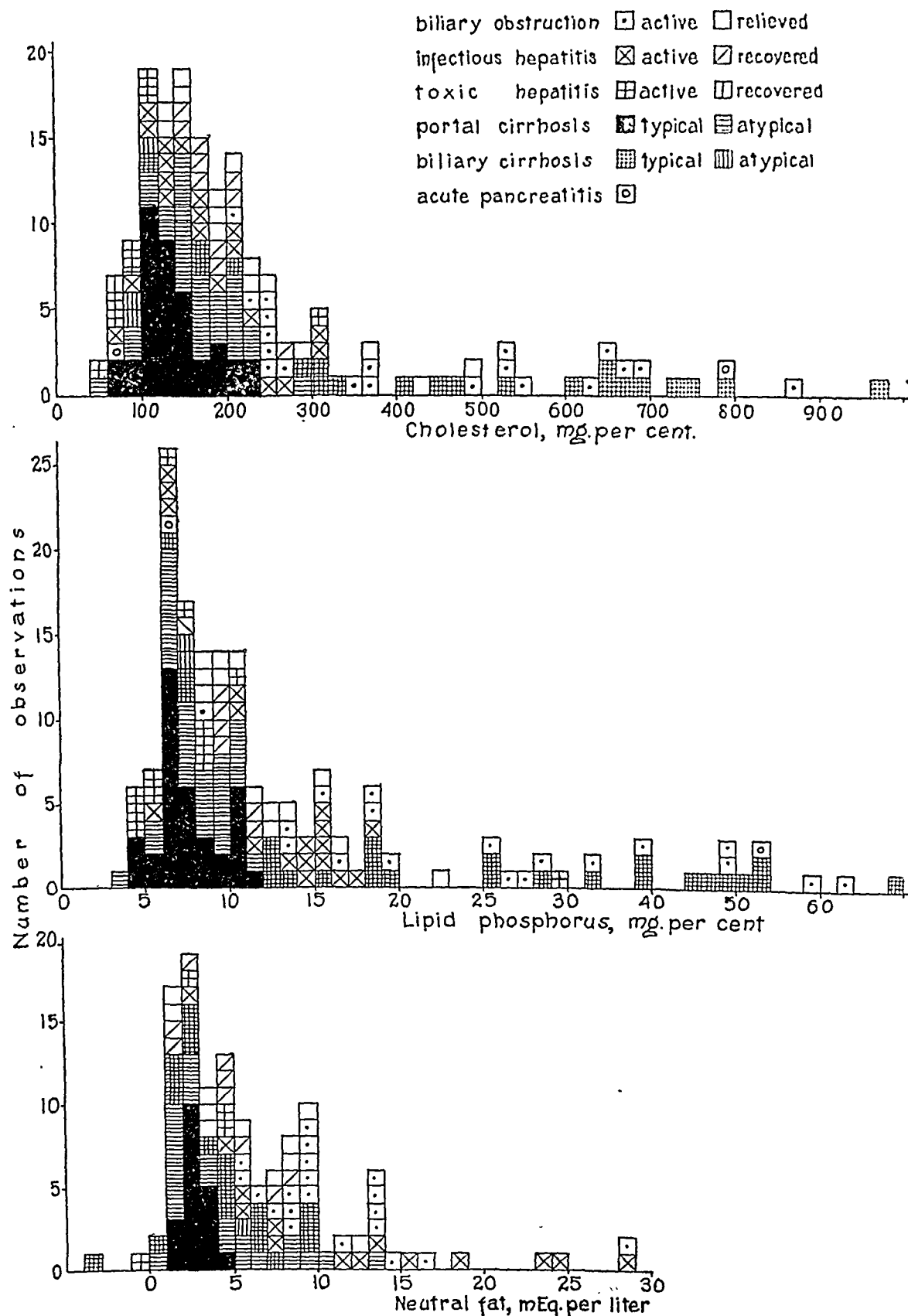
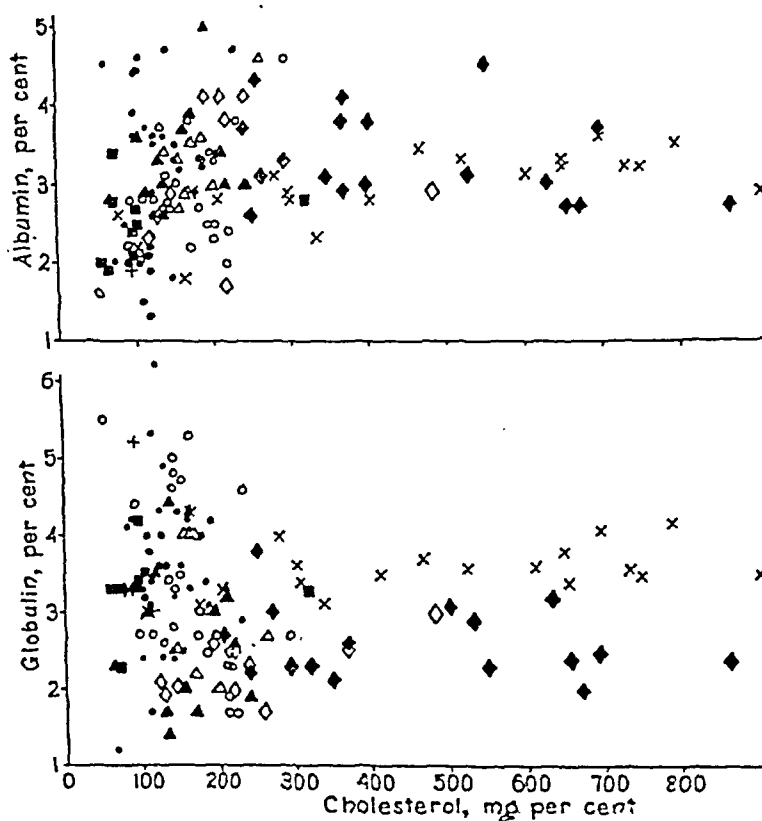


FIG. 1. CHOLESTEROL, LIPID PHOSPHORUS AND NEUTRAL FAT IN THE SERUM OF PATIENTS WITH VARIOUS TYPES OF LIVER DISEASE

extreme cachexia or with extensive destruction and degeneration of the parenchyma of the liver.

In general, cholesterol was higher in the patients with infectious hepatitis than it was in those with toxic hepatitis. There were, however, distinct exceptions to this rule. In 2 of the cases with infectious hepatitis, values below 100 mgm. per cent were observed. One of these accompanied an acute postpartum nephritis. True hypercholesterolemia, values greater than 300 mgm. per cent, was encountered only twice, in conjunction with acholic stools. It might have been noted more frequently if patients had been seen earlier in their illnesses. Illustrative cases are shown in Figure 5. In 3 cases with somewhat similar courses, studied only after icterus had begun to improve, cholesterol lay within normal limits. At least 1 of these had acholic stools earlier. Of the 8 patients with normal or high cholesterol, only

1 died; her icterus was a terminal feature accompanying pneumonia. Of the 7 with hypocholesterolemia (cholesterol less than 150 mgm. per cent), on the other hand, only 1 survived. It was noted that this patient had icterus when he was admitted to the hospital immediately after he had been scalded. How long the jaundice had persisted is as uncertain as its origin. As it subsided the serum cholesterol rose from 66 to 173 mgm. per cent. This is the only uncomplicated infectious case in which a cholesterol lower than 100 mgm. per cent was observed. On the other hand, concentrations below 100 mgm. were encountered in 4 patients in the terminal stages of hepatitis caused by drugs and chemicals. In these, jaundice was a late phenomenon overshadowed by symptoms of hepatic insufficiency. A diagnosis of arsphenamine hepatitis and encephalitis was made on a negress who, after antisiphilic treatment, was admitted



Biliary obstruction, active \blacklozenge , relieved \circ ; infectious hepatitis, active \blacktriangle , recovered \triangle ; toxic hepatitis, active \blacksquare , recovered \square ; portal cirrhosis, typical \bullet , atypical \circ ; biliary cirrhosis, typical \times , atypical $+$.

FIG. 2. THE RELATION OF CHOLESTEROL TO ALBUMIN AND TO GLOBULIN IN THE SERUM OF PATIENTS WITH VARIOUS TYPES OF LIVER DISEASE

to the hospital with a temperature of 104.4° F. in a semistuporous condition, with a number of anomalous features, including x-ray evidence of a tumor in the sella turcica. Serum cholesterol when she was improving was 318 mgm. per cent, the icterus index was 50. Despite a distortion of the interrelationships between the lipid fractions indicating a disorder of the liver, the cephalin flocculation test was negative. The diagnosis in this case, therefore, seems doubtful. The data on the whole suggest that hypercholesterolemia in hepatitis is an indication that the inflammatory condition has involved the bile ducts, causing obstruction. Whether this is a distinctive feature of certain types or stages of hepatitis is a suitable subject for inquiry.

In patients with typical portal cirrhosis, chole-

sterol was usually normal or low. In the cases with atypical portal cirrhosis, it lay in the same general range, although the average concentrations were somewhat higher. This difference depends largely on the inclusion in this group of certain cases. The patient with calcified adhesive pericarditis, despite intractable ascites, hepatomegaly and splenomegaly, had a normal serum lipid pattern throughout. This case accounts for 7 of the cholesterols above 160 mgm. per cent. Two others are contributed by another patient with heart failure. Three are from the patient who had dilated pancreatic ducts, and 1 from 1 of the patients with previous cholecystectomy. In 1 instance, the cholesterol rose from 129 to 179 mgm. per cent under therapy. Among the typical cases, 3 of the cholesterols above 160 mgm. per cent are from a pa-

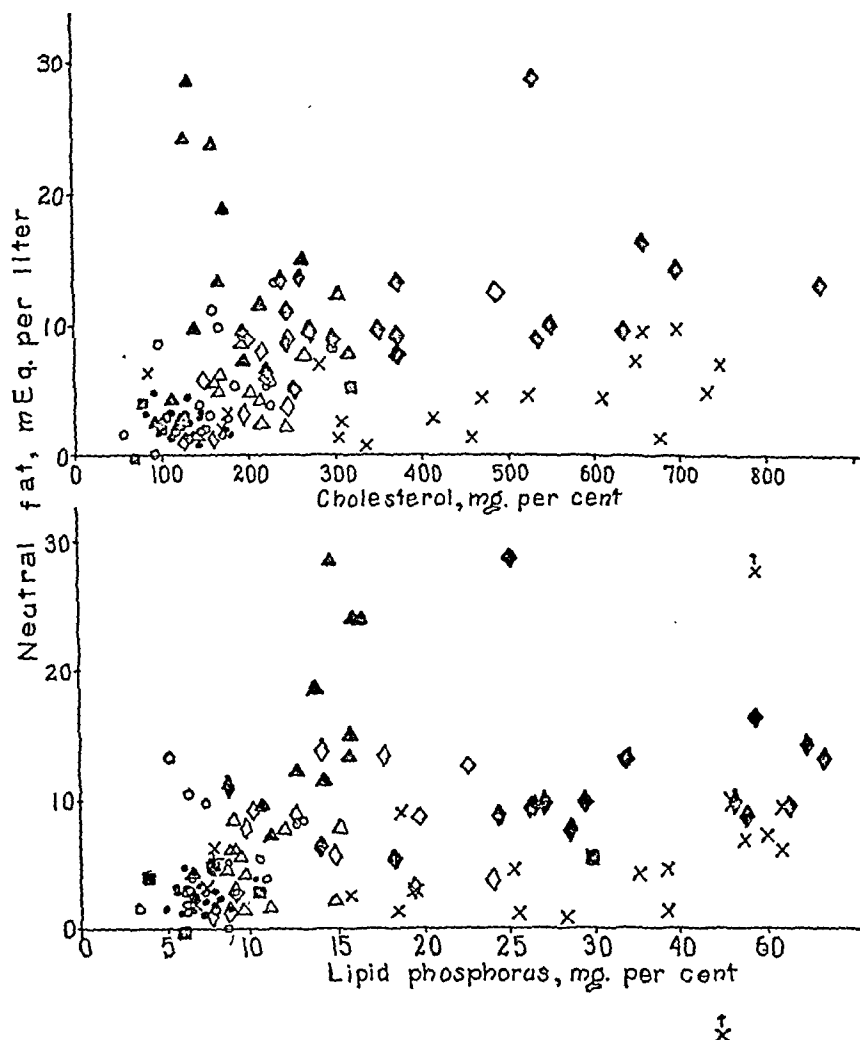


FIG. 3. THE RELATION OF NEUTRAL FAT TO CHOLESTEROL AND TO LIPID PHOSPHORUS IN THE SERUM OF PATIENTS WITH VARIOUS TYPES OF LIVER DISEASE

For interpretation of the symbols, see Figure 2.

tient who had successive values of 189, 185, 161, and 125 mgm. per cent. This probably illustrates the usual course of the disease. In 3 cases, however, the cholesterol rose in the course of the disease. In 1 instance a rise from 143 to 225 mgm. per cent marked a period of improvement in which ascites, previously requiring frequent paracentesis, was controlled by diet and diuretics. A rise from 154 to 208 mgm. per cent in another case attended symptomatic improvement. The third, when first seen, extremely malnourished, had a cholesterol of only 64 mgm. per cent. This rose to 112 and then to 229 mgm. per cent. Despite the fact that this woman died of a ruptured esophageal varix and suffered, besides, from heart failure, she never developed any considerable ascites. At autopsy, the liver was not characteristic of portal cirrhosis. Instead, there was a focal fibrosis, while the cells and architecture of most of the organ were well preserved.

The figures in biliary cirrhosis above 300 mgm. per cent are all from 1 patient who had also diabetes and necrobiosis (see Protocol 44700). Her course will be discussed in detail elsewhere. The patient with a cholesterol of 285 mgm. per cent also had a long-standing history of biliary stasis. Another patient with a similar condition had cholesterols of 167 and 177 mgm. per cent. It is known that cholesterol was 230 and 254 mgm. per cent $1\frac{1}{2}$ years and 1 year earlier, respectively. Another with an acute history had a cholesterol of 207 mgm. per cent 2 weeks before death. The 2 cases with cholesterol below 150 mgm. per cent died after prolonged courses, in cachectic states, 1 with extreme recurrent ascites. The condition of the atypical cases can be judged from the sketches of their records given in the protocols. It may be inferred that recurrent or partial biliary obstruction gives rise to hypercholesterolemia which diminishes as hepatic destruction progresses, ultimately giving way to terminal hypocholesterolemia, either as a result of the extreme malnutrition or the loss of liver parenchyma.

In this connection, although serum proteins may seem irrelevant to a discussion of lipids, they may throw some light on the effect of malnutrition. It must be recognized, however, that they are criteria of nutrition in a special sense only. Much emphasis has been placed upon hyperglobulinemia in cirrhosis. In the present series (see Figure

2A), serum globulin was below 3 per cent 7 times in typical portal cirrhosis, 4 per cent or above 14 times, and 3 to 4 per cent 9 times. Corresponding figures for atypical cirrhosis were 10 below 3 per cent, 6 above 4 per cent, and 9 between 3 and 4 per cent. In addition, globulin exceeded 4 per cent 5 times in biliary cirrhosis, 3 times in active acute hepatitis, 3 times after recovery, and once in biliary obstruction. Hyperglobulinemia is not, therefore, a reliable diagnostic sign of portal cirrhosis. Serum albumin is reduced (less than 4 per cent) in most patients with liver disease (see Figure 2B). Although the lowest figures coincide with low cholesterol, there is no direct correlation between the 2 variables, nor is albumin consistently depressed in any particular disease. It is probably determined rather by the nutritive state of the patient.

Although attention in the past has been confined chiefly to cholesterol, phospholipid (12) is quite as much disturbed in diseases of the liver. In general, the 2 follow one another, rising and falling together. Figure 1 suggests that diseases are, perhaps, a little more sharply differentiated into categories by means of phospholipids than by cholesterol. The parallelism between phospholipids and cholesterol is well illustrated in Figures 4 and 5.

About the concentration of neutral fat in the serum, both in diseases and in experimental disorders of the liver, little can be found in the literature (12). Statements about this lipid component are based chiefly upon determinations of total lipids or total fatty acids. The latter are peculiarly unreliable measures of neutral fat in conditions in which, as in liver disease, the concentrations of phospholipids and cholesterol esters are greatly altered. From Figure 1, it appears that concentrations of neutral fat are not high in the serum of most patients with liver disease. In only 2 instances did neutral fat rise to the maximum encountered in normal subjects (4). Neutral fat is, however, high in biliary obstruction and in infectious hepatitis, as compared with cirrhosis and toxic hepatitis in which it is usually comparatively low. From Figures 4 and 5, it will be seen that in both biliary obstruction and in some cases of infectious hepatitis neutral fat rises to fall again as the obstruction is relieved or the infection subsides. Neutral fat is, of course, estimated on the

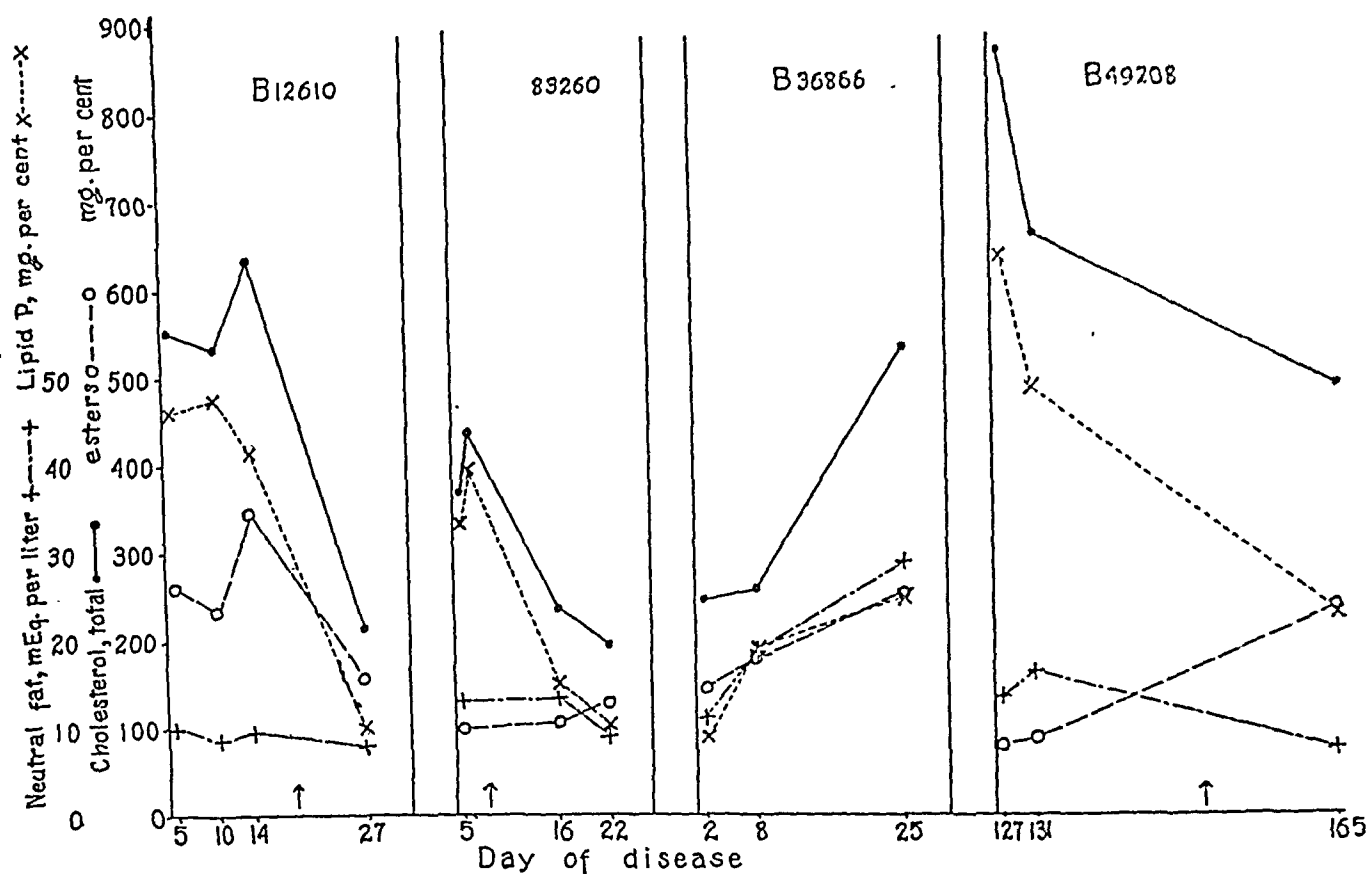


FIG. 4. THE COURSE OF THE SERUM LIPIDS IN PATIENTS WITH BILIARY OBSTRUCTION
For interpretation, see text.

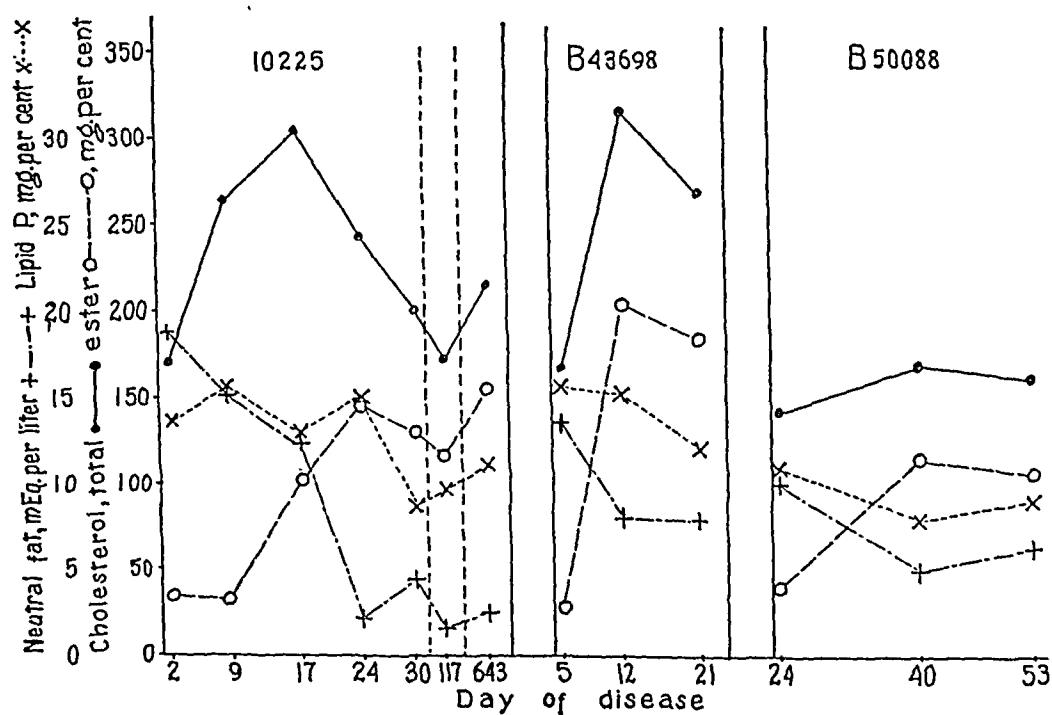


FIG. 5. THE COURSE OF THE SERUM LIPIDS IN PATIENTS WITH INFECTIOUS HEPATITIS
For interpretation, see text.

assumption that the proportions of phospholipids remain constant. If, as lipid phosphorus rose, the increments consisted entirely of phospholipids with 2 fatty acids, while those with 1 fatty acid diminished, fatty acids could increase out of proportion to phosphorus without addition of neutral fat. Even if it were assumed that every phosphorus were combined with 2 equivalents of fatty acid, the neutral fat figures in this study would not be significantly altered. If, on the other hand, the phospholipids with only 1 fatty acid increased disproportionately, neutral fat would be much greater than Figure 1 indicates in patients with high lipid phosphorus.

Some idea of the effect this would have can be gained from Figure 3 in which neutral fat is compared with lipid phosphorus and cholesterol. There is a general tendency for all 3 fractions to vary together, but the correlation is not consistent. The most striking exception is the patient (44700) with chronic biliary tract disease, diabetes, and necrobiosis who is responsible for all the diagonal crosses above 300 mgm. per cent of cholesterol and 15 mgm. per cent of lipid phosphorus. There is reason to believe that neutral fat in this case was underestimated, since on 1 occasion calculation yielded a value of -8.5 m.eq. per liter of fatty acid. The general tenor of the data would not be altered if the neutral fat figures of this patient and those with obstructive jaundice were raised. It would still remain true that elevation of neutral fat is confined almost entirely to patients with obstructive jaundice and infectious hepatitis. Among the latter it is those with early intense jaundice that are chiefly affected. The highest figures of all, however, were observed in the patient with postabortal *E. coli* septicemia. From Figures 1, 3, 4 and 5 it can be seen that neutral fat rose proportionally more in hepatitis than it did in obstructive jaundice. The only case of cirrhosis with neutral fat greater than 10 m.eq. per liter was the patient who had atypical portal cirrhosis with dilated pancreatic ducts.

The interrelationships of the serum lipids

It has been reported repeatedly that the ratio of free to total cholesterol is frequently elevated in hepatic disease (8, 9). Published data have, however, been obtained almost entirely by colorimetric

procedures that yield higher and more variable ratios than does the digitonin method employed for the present study. For example, one worker (8) gave as the normal range of the cholesterol ratio 0.30 to 0.50. On the other hand, in a large series of normal subjects several workers (13 to 15, 4) found by digitonin methods that the ratio, free cholesterol:total cholesterol, never departed from the narrow limits of 0.24 to 0.32. Furthermore, this relation was not disturbed in diseases of the thyroid gland (6), nephritis (16), and a variety of other disorders, in spite of the fact that these conditions greatly affected the concentration of cholesterol.

In Figure 6, total cholesterol and cholesterol esters of the patients of our series are compared. In only a few of the patients with cirrhosis is the ratio of esters to total cholesterol normal; in the others, it is invariably low. The grouping of points on the chart indicates that the distortion arises largely from a deficiency of ester forms. In only a few instances, when total cholesterol is greatly increased, does the concentration of esters exceed the upper normal limits; sometimes esters are almost extinguished. In patients with biliary obstruction and extreme hypercholesterolemia both fractions are sometimes elevated. In this condition and in acute hepatitis and biliary cirrhosis, the ratios are particularly reduced. From Figures 4 and 5, it can be seen that cholesterol esters fall at the onset of obstruction or acute hepatitis, even before total cholesterol rises. Conversely, during recovery, esters rise and the ratio is restored before the total cholesterol falls. This is particularly evident in B49208, Figure 4. Before operation, ester cholesterol was only 80 and 85 mgm. per cent, with total cholesterol of 782 and 572 mgm. per cent. Normally, with cholesterol esters as low as this, total cholesterol could not exceed 118 and 125 mgm. per cent. Fifteen days after operation, with relief of the obstruction, the esters had risen to 236 mgm. per cent when the total cholesterol had fallen to only 485 mgm. per cent. It is this tendency for the ratio to approach normal before the concentration does that accounts for the obstruction and hyperlipemic acute hepatitis cases that have normal ratios in Figure 6.

In those patients who develop hypercholesterolemia, free cholesterol rises strikingly at the onset, as esters diminish. At the height of the hyper-

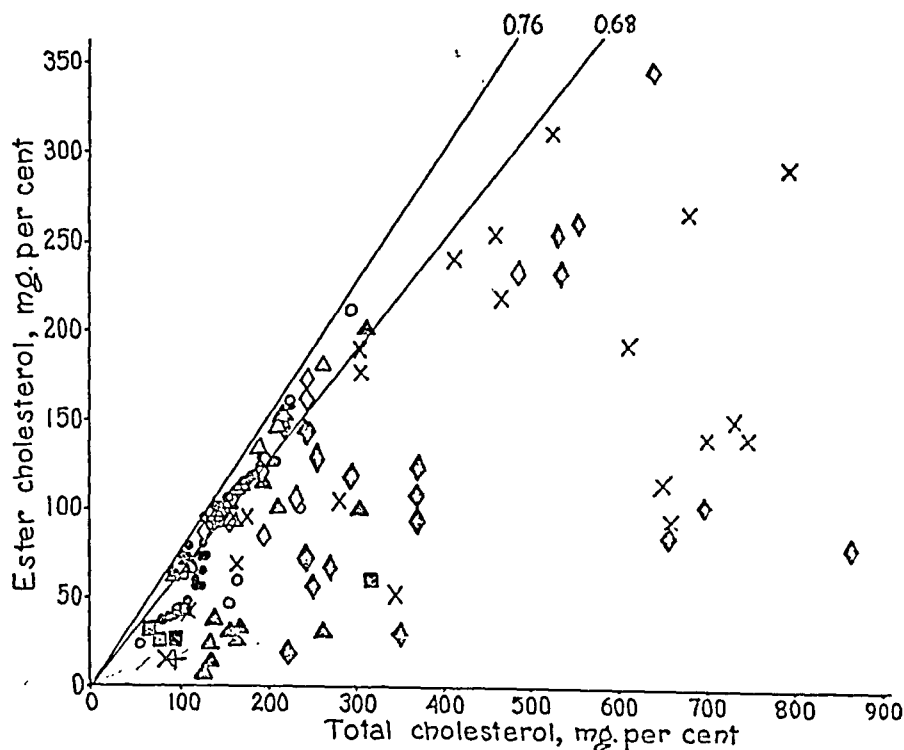


FIG. 6. THE RELATION OF CHOLESTEROL ESTERS TO TOTAL CHOLESTEROL IN PATIENTS WITH LIVER DISEASE

The diagonal lines defining ratios of 0.76 to 0.68 bound the zone of normal variation. For interpretation of the symbols, see Figure 2.

Solid circles = typical, open circles = atypical portal cirrhosis. Diagonal crosses = typical, vertical crosses = atypical biliary cirrhosis. Solid triangles = active, open triangles = recovered infectious hepatitis. Solid squares = active, open squares = recovered toxic hepatitis. Solid diamonds = active, open diamonds = relieved biliary obstruction.

lipemia, free cholesterol, which is normally a small fraction of the total, may exceed the normal value for total cholesterol. In 1 instance, of a total of 730 mgm. per cent, 530 mgm. per cent was in the free form. In patients with extreme hypocholesterolemia, on the other hand, free cholesterol, though high in proportion to the esters, may lie below the normal limits.

Just as the ratio of free cholesterol to total cholesterol is more constant than the concentration of total cholesterol or either of its fractions in the serum of normal persons, so the proportion of phospholipid to cholesterol is less variable than is the concentration of either phospholipid or cholesterol (6). The ratio, cholesterol:lipid phosphorus, however, is not constant, as is the ratio of free to total cholesterol, but increases with the cholesterol in a manner that has been described in an earlier paper (6).

In liver disease, lipid phosphorus, in general, parallels total cholesterol. The normal relations

between the 2 are not, however, preserved. In most cases, the ratio of cholesterol to lipid phosphorus is lower than normal. Especially in the hyperlipemic cases, this distortion implies that phospholipids rise proportionally more than total cholesterol. In a review of the normal relation between these 2 lipid fractions, it was discovered that when lipid phosphorus is plotted directly against cholesterol, the distribution of points above 100 mgm. per cent of cholesterol is indistinguishable from a straight line. The distribution of points in thyroid diseases coincides with the distribution in normal persons or a rectilinear extension of this distribution. The best straight line calculated from 856 observations in which cholesterol varied from 108 to 911 mgm. per cent, was defined by the equation:

$$\text{lipid phosphorus} = 0.0294 \text{ cholesterol} + 3.62, \\ \text{S.D.} = \pm 1.043 \text{ mgm. per cent}$$

This line meets the y axis at a point indicating that

cholesterol should disappear when the lipid phosphorus falls to 3.62 mgm. per cent. Actually cholesterol does not disappear, but falls at a slower rate than lipid phosphorus when the latter approaches this concentration. The curvilinear relation of the ratio, cholesterol:lipid phosphorus, to cholesterol, defined in an earlier paper (6) arises from the fact that the line defining the relations of these 2 fractions does not pass through the origin. By means of the linear relation defined by the new equation, it is possible to construct a graph somewhat like that used for the evaluation of the cholesterol ratio. This has been done in Figure 7. The radial lines define ratios of lipid phosphorus — 3.62 to total cholesterol. The lines defining the normal limits, with slopes of 0.0205 and 0.0383 respectively have not as precise significance as those defining the limits of the cholesterol ratio, because the latter is more constant than the ratio of lipid phosphorus to cholesterol. These lines are drawn to include twice the mean deviation of the ratio at 200 mgm. per cent of cholesterol, which is about ± 0.009 . The deviations are smaller at low than at high concentrations of cholesterol, which justifies, to some extent, the use of radial lines.

It is evident from Figure 7 that the ratio, lipid phosphorus:cholesterol, is unmistakably larger

than normal in a large proportion of patients with liver disease. The most striking elevations are found in subjects with biliary obstruction and acute hepatitis. Certain exceptions are to be found. Among these are patients on the road to recovery from obstruction or hepatitis. This ratio, like the cholesterol ratio, seems to return to normal more rapidly than the concentrations of lipids do. Other exceptions are subjects with cholesterol below 100 mgm. per cent. To these, for reasons given above, the chart does not properly apply. The cirrhosis cases, for the most part, have normal ratios.

In Figure 8, the ratio of free to total cholesterol is compared with that of lipid phosphorus to cholesterol. The horizontal and vertical lines define the normal limits of the 2 ratios. Area 5, therefore, presents the normal zone. In this area, lie patients who have recovered from acute hepatitis or have been relieved of biliary obstruction and a few cases of typical or atypical cirrhosis. There are no points in Areas 1, 4, and 7 which represent cholesterol ratios less than 0.24. Area 2 is also clear. This means that the ratio of lipid phosphorus to cholesterol is not altered if the cholesterol ratio is normal. Biliary obstruction and acute hepatitis tend to congregate in Area 3,

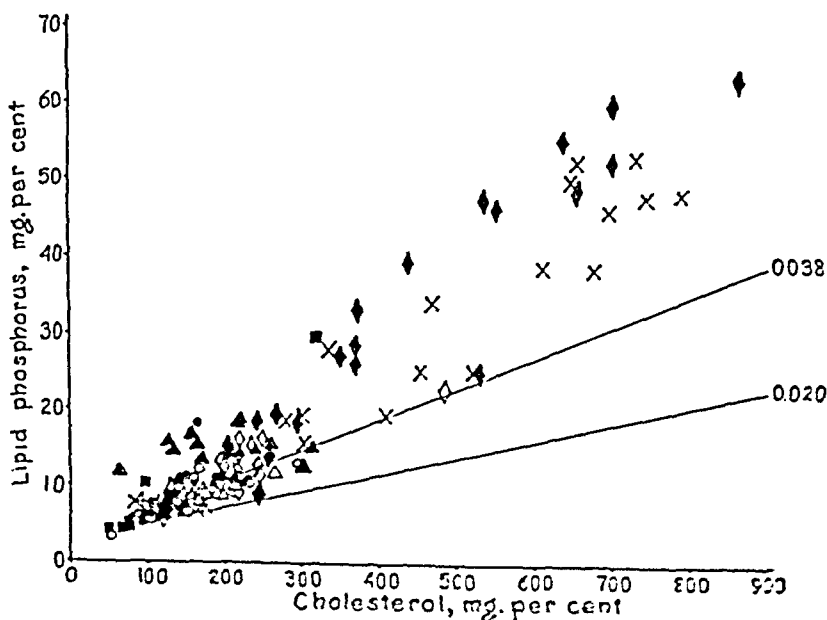


FIG. 7. THE RELATION OF LIPID PHOSPHORUS TO CHOLESTEROL.

The diagonal lines bound the zone of normal variation of the ratio of lipid phosphorus—3.62 to cholesterol. For interpretation of the symbols, see Figure 6.

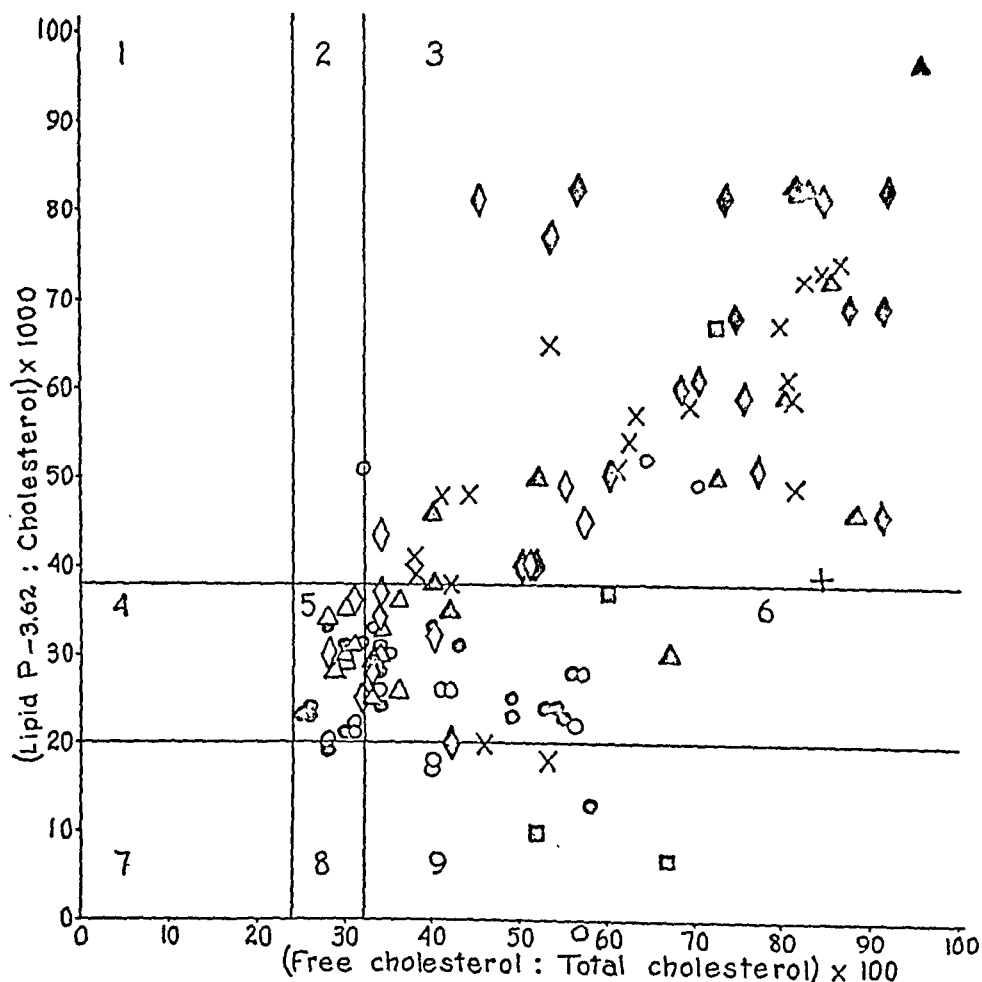


FIG. 8. THE RELATION OF THE LIPID PHOSPHORUS: CHOLESTEROL RATIO TO THE CHOLESTEROL RATIO IN PATIENTS WITH LIVER DISEASE

The vertical and horizontal parallel lines define the normal limits of variation to these ratios. For interpretation of the symbols, see Figure 6.

where both ratios are high. In this same area appear some subjects with atypical cirrhosis. As acute hepatitis and obstruction improve they frequently move through Area 6; that is, the lipid phosphorus:cholesterol ratio is restored earlier than the cholesterol ratio. In this area are also found most of the observations on typical and atypical cirrhosis. The few points in Areas 8 and 9 are almost all derived from patients with extreme hypolipemia.

Lipotropic agents

Choline chloride, in doses varying from 10 to 25 grams a day, was given for varying periods to 3 patients presumably suffering from portal cirrhosis. In none of them did it have any demonstrable effect upon the clinical course nor upon the serum lipids. Two of these subjects developed diarrhea when they took choline. This reaction

occurred in 4 of the 8 patients who received this compound, but in 3 the diarrhea was transitory. The compound was also given to 3 patients with acute hepatitis. All three recovered, but it is impossible to say that their clinical courses were influenced by the choline. Another patient (Tables I and II, 28639) was given 10 grams subcutaneously on the second and third days preceding her death.

The course of the lipids in the eighth patient who received choline is illustrated in Figure 9. This is the patient with diabetes, necrobiosis, and chronic icterus with partial biliary obstruction and cholangitis (Protocol 44700). The serum lipids were greatly increased throughout her course, with strikingly high ratios of free to total cholesterol and of lipid phosphorus to cholesterol. The lipid picture resembled that of obstructive jaundice, with the exception that neutral fat was

not elevated. Nevertheless, biliary obstruction was never complete. From the 96th to the 113th day in the hospital, she was given 1 gram of choline chloride daily. Under this treatment cholesterol and lipid phosphorus fell and continued to decline in the next 2 weeks without choline. At the same time, the lipid ratios fell towards normal. Choline was again resumed on the 128th day and was taken irregularly until the 167th day. On the 186th day, the serum lipids were almost normal. Subsequently they rose again, falling only shortly before death. No apparent improvement in the clinical condition accompanied the reduction of the serum lipids. Whether the latter can be attributed to the choline, it is impossible to state.

One of the patients with portal cirrhosis who had reacted to choline with diarrhea was given 3 grams of methionine daily for a month without any demonstrable change of clinical signs and symptoms or of serum lipids. Another patient who developed nephritis and hepatic enlargement with icterus during the puerperium, following

erysipelas, was given 5 grams of methionine daily for 4 days shortly before death.

Lipocaic was given to a patient who developed fatty diarrhea and multiple vitamin deficiencies after a subtotal pancreatectomy for pancreatic carcinoma that had completely obstructed the common bile duct. Although she took as large doses of the material as she was able (up to 12 capsules a day), together with pancreatin and vitamins, she went rapidly and uninterruptedly downhill until her death. During her postoperative course, her lipids, which had been depressed, rose to the normal range despite extreme wasting. The lipid ratios, however, remained abnormal throughout.

Liver analyses

Results of the liver analyses are shown in Table I. For comparison in the same table are analyses of livers from 3 patients presumably free from hepatic disease and other disorders known to affect serum lipids, and a summary of similar analyses published by Ralli (17). The milliequivalents

TABLE I
Lipids of the liver

Case number	Hours after death	Liver weight	Solids	Fatty* acids	Lipid phosphorus	Cholesterol				Neutral fat
						Total	Free	Ester	Free:Ester	
		grams	grams per kgm.	m.eq. per kgm.	grams per kgm.	grams per kgm.	grams per kgm.	grams per kgm.		m.eq. per kgm.
Liver disease										
B43797	5.5		319	532.0	0.963	2.40	1.27	1.13	0.53	473.2
B36812	16.0	4675	535	1327.0	0.729	2.05	1.43	0.62	0.70	1283.1
28639	12.5	2325	197	63.9	1.207	2.63	2.51	0.12	0.95	-6.5
A57138	22.5	2300	532	1036.5	0.642	6.76	2.96	3.80	0.44	958.4
19694	21.0	650	228	53.0	0.832	2.77	2.80	0.00	1.00	4.7
A47621	16.0	1525	225	190.6	0.690	3.09	2.57	0.52	0.83	149.1
B43658	6.5	1050	197	55.0	0.803	2.64	2.05	0.59	0.78	6.9
A45460	7.5	850	228	95.8	1.001	3.49	2.79	0.70	0.80	35.9
B43742	16.5		184	61.4	0.713	3.03	2.70	0.33	0.89	19.1
Normal										
6269	19.0	2045	297	219.9	1.139	3.40	2.09	1.31	0.62	179.3
6270	8.0	1650	274	188.5	1.180	3.74	2.59	1.15	0.69	116.9
6366	14.0	1450	284	183.7	1.284	2.97	2.26	0.71	0.76	167.1
Ralli's (17) normals										
Minimum				59.1	0.560	2.40	1.97	0.43	0.92	27.7
Maximum				264.0	1.167	3.88	2.17	1.71	0.56	141.5
Average				128.0	0.893	2.83	2.04	0.79	0.72	79.7

* Concentrations of all lipid components are referred to weight of fresh tissue.

† In terms of fatty acids.

TABLE II
Serum lipids of patients with liver analyses

Case number	Date	Serum protein		Fatty acid	Lipid phosphorus	Cholesterol			Lipid phosphorus -cholesterol	Neutral fat*
		Total	Albumin			Total	Free	Free:Total		
		per cent	per cent	m.eq. per liter	mgm. per cent	mgm. per cent	mgm. per cent			m.eq. per liter
B43797	1943									
	June 21	5.15	3.08	26.2	27.3	350	320	0.91	0.019	9.6
	July 20	4.94	2.81	9.5	7.2	138	46	0.33	0.068	2.9
B36812	Jan. 21	8.19	2.93	18.4	12.3	166	106	0.61	0.052	9.7
	Feb. 1	6.22	2.72	18.2	11.1	155	108	0.70	0.019	10.5
	Feb. 3			21.8	10.2	234	133	0.57	0.028	13.3
28639	May 29	5.00	3.30	37.5	14.5	132	108	0.82	0.082	28.5
	May 30	4.64	3.28	36.2		133	120	0.90		
	May 31	5.03	3.01	34.2	16.7	157	127	0.81	0.083	23.7
	June 1			33.6	15.8	127	120	0.95	0.096	24.2
A57138	1944 Nov. 18	5.46	3.92	10.4	7.9	91	47	0.52	0.042	5.0
19694	1943 Mar. 5	6.62	3.37	7.1	4.2	78	52	0.67	0.007	4.0
A47621	Jan. 12	6.14	2.03	6.5	5.6	79	42	0.53	0.024	3.2
	Jan. 26	8.39	2.18	7.5	7.7	122	49	0.40	0.033	3.0
B43658	June 4	5.85	2.91	6.1	5.5	104	42	0.40	0.018	2.9
A45460	1944 Nov. 14	6.25	1.77	7.4	6.1	139	63	0.45	0.018	1.9
B43742	1943 Apr. 3	7.08	1.90	10.3	7.7	93	78	0.84	0.044	5.4

* In terms of fatty acid.

of fatty acid are calculated from this worker's figures, which are given in grams, by assuming that the fatty acids were composed of equal parts of stearic, oleic, and palmitic acids. The values for lipid phosphorus have been recalculated by means of the factor by which she converted lipid phosphorus to grams per cent of phospholipid (18). From these figures, neutral fat has been estimated by the formula used for the estimation of neutral fat from our own analyses. Her figures for neutral fat were not employed because they would not be comparable to ours. The results of analyses of sera from the patients whose livers were analyzed are shown in Table II. Abstracts of protocols of the cases are given at the end of the paper. The three "normal specimens" of liver were taken from the livers of: (1) a man of 50 years who committed suicide by taking cyanide, (2) a man of 31 years who died 6 hours after a coronary occlusion, (3) a man of 29 years who died from a stab wound of the heart.

Interpretation of analyses of tissues from human autopsy material presents several knotty problems. In the first place, it is not possible to secure the specimens in such a fresh state that autolytic changes can be excluded. Fortunately, lipids appear to be less subject to autolysis than carbohydrate and proteins are. Ralli (17) found that total cholesterol and fatty acids did not change, though lipid phosphorus decreased slightly in a dog's liver kept in the refrigerator for 23 hours. The differences in the "normal" analyses in Table I are not related to the interval between death and examination. It is difficult to obtain suitable normal control specimens. The 3 analyses in Table I are not sufficient to establish the range of normal variation. In composition, however, all three are relatively uniform. Ralli's (17) figures for all constituents are far more variable, and her series of analyses is far larger than ours. It may be that her material is more representative or that ours is more fortunately

selected. In the general magnitude of all fractions, the two sets of analyses agree. Many others have reported liver analyses that differ widely from hers (17, 19) and ours, presumably because of the analytical techniques employed (20 to 23). It may be assumed that the structure of the livers of adults is relatively uniform, that the proportions of liver cells to connective tissue and other elements are approximately the same. It is quite otherwise in diseased livers. In cirrhosis, for example, the major part of a sample may consist, not of liver cells, but of undifferentiated connective tissue. The proportion of liver cells to connective tissue cannot be estimated, but it must

have a great influence upon the chemical composition of the specimens taken for analysis.

There is no feature common to all the pathological livers; but the most frequent abnormality is a relatively low concentration of phospholipids. The first, second, and fourth cases are distinguished from all the others by the high proportions of solids they contain. This is associated with the presence of excessive quantities of fatty acids, of which an unusually large fraction belongs to neutral fats. In B43797 and B36812 pancreatic tissue was almost entirely destroyed, though neither had diabetes. These presumably are examples of true dietary fatty livers comparable to those that de-

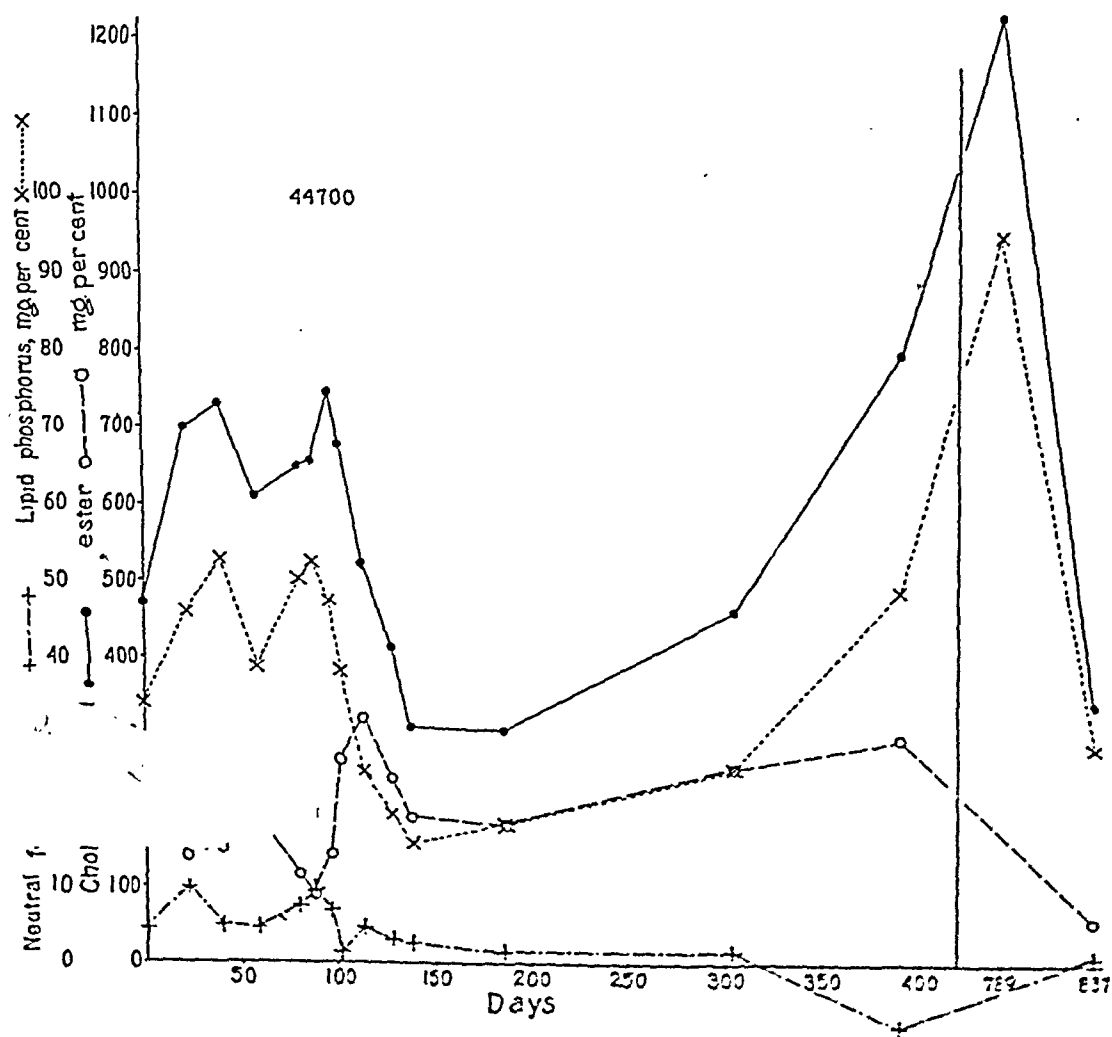


FIG. 9. THE COURSE OF THE SERUM LIPIDS OF A PATIENT WITH PARTIAL BILIARY OBSTRUCTION, CIRRHOSIS, DIABETES AND NECROSIS AND THE REACTION OF THE LIVER TO CHOLINE

For interpretation, see text.

velop in depancreatized animals treated with insulin. Phospholipids are reduced in both. Total cholesterol is also low, the deficiency affecting chiefly the free cholesterol fraction. B43797, when first observed, had high serum cholesterol, phospholipid, and moderately-elevated neutral fat in her serum, all of which fell before death, possibly because she had a biliary fistula. Cholesterol, lipid phosphorus, and neutral fat rose steadily in the serum of B36812, and the cholesterol ratio was high throughout. An erroneous diagnosis of portal cirrhosis was made because of an alcoholic history, a large liver, ascites, and high serum globulin. The serum lipid pattern, however, differs widely from that usually seen in portal cirrhosis, which is characterized by low concentrations of lipids and little distortion of the cholesterol ratio.

In some respects A57138 resembles the 2 preceding cases: total solids, fatty acids, and neutral fat are extremely high, lipid phosphorus greatly reduced, but cholesterol is enormously increased by accumulation of esters. This corresponds closely to the pattern of lipids described in the livers of animals with purely dietary fatty livers. Certainly from his history, the patient was entitled to such a condition. It is, however, impossible to evaluate or to discount the poisonous effects of the rubbing alcohol and the renal tubular destruction.

All the other livers of the series contain less than the normal complement of solids and in all but one, A47621, the deficiency can be attributed partly to a reduction of the concentration of fatty acids in the organ. Fatty infiltration in a gross sense is not, therefore, a consistent feature of hepatic disease. Case 28639 is peculiarly intriguing. Whether as a result of some unknown drug or puerperal sepsis, the patient had jaundice and other evidences of profound injury to the liver, is not known, and post mortem examination revealed no gross nor microscopic disturbance of hepatic structure. The chemical composition of the organ is profoundly altered, as might have been surmised from the serum analyses. The most striking change in both serum (last observation) and liver is the almost complete extinction of cholesterol esters and the relative preservation of the phospholipids. In fact, serum lipid phosphorus is high. This patient received choline for the last 3

days of her life. On the other hand, no neutral fat could be detected in the liver, although its concentration in the serum is extremely high, as if its utilization had been blocked. The calculation of neutral fat is, of course, subject to error because the composition of the phospholipids is unknown. If these contained an unusually large proportion of compounds containing only a single fatty acid the neutral fat would be elevated somewhat.

The features common to this liver and that of 19694, who died of a toxic hepatitis, are the absence of cholesterol esters and the deficiency of neutral fat. Phospholipid is also low in 19694. All the lipid components in his serum were also reduced. These distinctions may arise from differences in the duration of the disease. Case 28639 died only a week after the onset of her illness, while 19694 had been exposed for months to the poison which killed him and had jaundice for at least 3 weeks before his death.

The 4 remaining patients died with cirrhosis of various degrees and kinds. The low solids and lipids cannot, therefore, be attributed altogether to changes in composition of the liver cells; replacement of these cells by connective tissue must be a contributory factor. The lipid constituents are not, however, uniformly affected. The concentrations of total cholesterol are quite well preserved. If the reductions of lipid phosphorus and fatty acids derive from replacement of liver cells by connective tissue, the liver cells must contain excessive amounts of cholesterol. Both morphologically and chemically the livers differed from one another. The most classical picture of portal cirrhosis was seen in B43658. With the exception of neutral fat, which is disproportionately low, the interrelationships of the lipid components in his liver are relatively well retained. The pattern of lipids in the liver of A45460 is very much the same, except for larger proportions of cholesterol and neutral fat. Both these patients had the characteristic hypolipemia of portal cirrhosis.

When allowance is made for the fibrous tissue in the liver of A47621, neutral fat and cholesterol appear to be elevated, while lipid phosphorus is depressed. Much central necrosis was found in this liver. The cholesterol ratio in both serum and liver was greatly elevated in B43742, whose liver showed a more diffuse fibrosis than the others with some evidence of bile-stasis.

DISCUSSION

Although the amount of work involved in these analyses is large, the definite conclusions that can be drawn from them are comparatively meager. The material studied was so large and varied that no single condition was exhaustively examined. The investigation can be regarded only as a preliminary survey. The results are highly suggestive. They raise the hope that possibly such partitions of lipids may prove a more accurate means than the rather empirical tests thus far employed for the differentiation of hepatic disease and disorders. They may give more information than measurements of cholesterol and its fractions, to which attention has been largely confined. This is to be expected from experimental studies of dietary fatty livers and toxic hepatitis, in which it has been found that the formation of a balanced mixture of phospholipids is essential to the proper function of the liver and the orderly metabolism of fat (3).

Obstruction of the biliary passages appears to lead regularly to the accumulation in the serum of cholesterol, of phospholipids and, to a lesser extent, of neutral fat. The character of the cholesterol is also altered. Instead of consisting chiefly of esters, free forms predominate; esters are not only relatively, but absolutely, reduced. Available information indicates that cholesterol esters occur in the cells of only those organs or tissues in which there is active sterol metabolism: the liver, testes, adrenals, etc. This suggests that the metabolism of cholesterol is linked with the formation of esters. It has now been demonstrated that cholic acid is produced from cholesterol (24). In addition, large amounts of cholesterol are poured into the bile, chiefly in the free state. It is conceivable that obstruction to the escape of bile backs up these materials and retards the processes by which they are formed in the liver. This would explain the accumulation of free cholesterol in the serum and the reduction of esters. It has been demonstrated that administration of excessive amounts of cholesterol to rats is one of the most effective methods of producing fatty livers (25 to 29). The proportions of cholesterol esters in the livers of such rats are unusually large (25 to 29), while in the blood serum they are reduced (30, 31). The formation of phospholipids is dis-

turbed, and the turnover of these compounds in the liver is retarded in this condition (32, 33). Cholesterol may compete with phospholipids for suitable fatty acids. Both cholesterol esters and phospholipids in proper proportions may be required for the orderly conduct of hepatic lipid metabolism. Whether all the phospholipid components of the serum are equally disturbed or whether the proportions of these components are altered by biliary obstruction remains to be determined. If the condition is similar in origin to the cholesterol fatty liver, it should respond to the administration of choline and other lipotropic factors (34). Some workers (35, 36) have, indeed, shown that in experimental biliary obstruction fatty infiltration of the liver can be minimized by the administration of high protein diets.

When the parenchyma of the liver is profoundly insulted by poisons or other destructive agents, the serum lipids appear to decrease. At the same time, the pattern of lipids is distorted in much the same manner as it is in biliary obstruction. This suggests that the maintenance of proper concentrations of lipids in the serum depends upon the presence of an adequate quantity of liver tissue, while the preservation of the normal pattern of lipids depends on the orderly function of the liver cells. This hypothesis would explain the fact that in cirrhosis concentrations of lipids are usually low, while patterns are little disturbed. Although the quantity of functioning liver tissue in cirrhoses is greatly reduced, the remaining cells may be comparatively normal. Distortions of patterns observed in particular cases would suggest that in these there is a more active process at work. It may be of some significance that such distortions were encountered more frequently in patients with atypical than they were in patients with typical cirrhosis.

In hepatitis 2 distinct types of disorders were seen. Certain cases had initial hyperlipemia, resembling in character, but seldom equalling in magnitude, that of obstructive icterus. Increases of cholesterol and lipid phosphorus were more moderate, while increases of neutral fat were greater in hepatitis. In other patients with infectious hepatitis and in the patients with toxic hepatitis, cholesterol and lipid phosphorus were low with an extreme deficiency of cholesterol esters. Clinical evidence suggests that the hyper-

lipemic cases had a greater degree of biliary obstruction than the others. If the hypotheses advanced above are sound, this should yield a hyperlipemia like that of biliary obstruction, but of smaller magnitude because of the injury to the liver cells. It is possible that the difference between the hyperlipemic and hypolipemic cases may depend upon etiological factors; it may prove to have prognostic significance.

The experiments with lipotropic agents offer little encouragement to the use of these agents, although they may have been of some benefit to 5 of the patients. This does not mean that these agents may not be useful therapeutic aids, but only that the cases or the dosage may have been improperly selected. There is experimental evidence that choline or methionine may prevent or alleviate the hepatic lesions produced by certain liver poisons and diseases (1, 2, 37). These and other lipotropic agents will preserve the lives of pancreatectomized animals (38, 39). They will prevent or eliminate fatty infiltrations that result from dietary deficiencies. It has been suggested that similar fatty infiltrations characterize diseases of the liver arising from other causes. The series of liver analyses is all too small to warrant any generalizations or attempts to correlate lipid patterns of serum and liver closely. Like the serum analyses, it suggests that such correlations may be found by further studies of a similar nature. The diversity of the disorders in both sera and livers may explain the inefficacy of choline and other lipotropic agents in the cases to which they were given in this study. The benefits that may accrue from adding chemical analyses to morphological techniques is especially evident in such cases as 28639. This is more feasible with respect to lipids than to other compounds, since the lipids appear to be comparatively stable. It should be particularly informative when applied to the liver because this organ plays such an important rôle in the metabolism of lipids.

SUMMARY AND CONCLUSIONS

The serum lipids have been fractionated on 174 occasions in 70 patients with diseases of the liver and bile ducts.

Total cholesterol and lipid phosphorus are elevated in the serum of patients with biliary ob-

struction, returning to normal when the condition is relieved by operation. They are also high in a certain proportion of patients with biliary cirrhosis and with infectious hepatitis. The latter group is composed chiefly of subjects who have had intense jaundice at the onset of their illness, with acholic stools. This suggests that hyperlipemia is a product of biliary obstruction.

Cholesterol and lipid phosphorus are normal or subnormal in most patients with portal cirrhosis, tending to fall as the disease advances. In patients with toxic hepatitis and some with infectious hepatitis, these lipid components are also reduced. This suggests that hypolipemia arises from extensive degeneration or destruction of liver parenchyma.

Neutral fat in liver disease seldom rises above the upper normal limits. It is, however, usually above the average normal, and occasionally greatly elevated, in obstructive icterus and infectious hepatitis, falling when obstruction has been relieved or when the hepatitis subsides.

The most frequent disorder of serum lipids in liver diseases is an increase of the ratio of free to total cholesterol. Free cholesterol is usually not only relatively but absolutely increased, while there is usually a deficiency of esters.

The relation of phospholipids to cholesterol in normal subjects has been redefined. In liver disease, especially in those disorders attended by hyperlipemia, the ratio of lipid phosphorus to cholesterol is greater than normal.

The two ratios, free cholesterol:total cholesterol and lipid phosphorus:total cholesterol, are differently and somewhat characteristically altered in various diseases of the liver.

Choline chloride and other lipotropic agents were given to a small series of patients with diseases of the liver, without definitely beneficial effects.

The lipids of the sera of 9 patients have been compared with the lipids of samples of liver obtained at autopsy.

PROTOCOLS

44700, in addition to her hepatic condition, had diabetes and necrobiotic lesions of the extremities. These began as purplish nodules, the centers of which became necrotic and later ulcerated. Cultures of the necrotic material before ulceration were sterile; biopsies of the ulcers revealed

only chronic inflammation. On the legs, the ulcers became confluent and quite extensive. Two years before she came to the New Haven Hospital, she had a period of intermittent jaundice without abdominal pain. Her liver was considerably enlarged. She died after enterostomy for symptoms suggesting intestinal obstruction. Post mortem examination revealed chronic cholecystitis and both extra- and intrahepatic cholangitis, stones partially obstructing the common bile duct, adhesions and abscesses around the gall-bladder, and abscesses in the liver. During her course of $2\frac{1}{2}$ years in the hospital, she had jaundice of variable intensity, usually slight. The diabetes was satisfactorily controlled.

B43797, female, born in 1875. For 20 years before admission on June 14, 1943, she had suffered from attacks of right upper quadrant pain, distention and eructations, lasting 1 or 2 days. Three weeks before admission she had sharp pain in the same area, followed shortly by chills, fever, jaundice, light-colored stools, and dark urine. On admission the temperature was 103.4° F., the liver considerably enlarged and tender with a mass below it suggesting the gall-bladder. At operation on June 21, a hard tumor of the pancreas was reported and a cholecystojejunostomy was performed. Because fever and symptoms did not subside, an external choledcho-duodenostomy was performed on July 20. She died 4 days later with a biliary fistula. Autopsy revealed a carcinoma of the pancreas which had obstructed the common bile duct and invaded the liver. No normal pancreatic tissue except a few islands of Langerhans could be found.

B36812, female, born in 1914. In 1941, she was admitted to another hospital because of excessive menstrual bleeding, fever, and icterus. January 19, 1943, she was admitted to the New Haven Hospital because of increasing weakness and insomnia, continued menorrhagia, with tingling and numbness of the extremities, obviously alcoholic. She confessed to drinking a pint of whiskey a day but contended that she had developed this habit only after the attack of jaundice mentioned above. Because of the alcoholism, a moderately-enlarged liver, ascites and telangiectases, a diagnosis of portal cirrhosis was made. The cephalin flocculation test was 4 plus, the prothrombin time normal, there was 28 per cent retention of bromsulfalein at the end of 30 minutes, and she was slightly icteric. She was readmitted March 25, in shock, almost exsanguinated from vaginal bleeding, and died before either diagnostic or therapeutic measures could be instituted. Post mortem examination revealed only the slightest fibrosis of the liver which was tremendous. The hepatic cells were profusely infiltrated with fat. The pancreas, except for some islands of Langerhans, was entirely replaced by fibrous tissue, the remnants of the ducts were dilated, and the main duct was occluded near its entrance into the duodenum, for no discoverable cause.

28639, female, born 1913. On May 25, 1943, the patient aborted after insertion of a catheter into the uterus. The abortion was followed by profuse hemorrhage. On May 27, she was admitted to the hospital in coma and shock, with a blood pressure of 50/30. She was given two 500

ml. transfusions of whole blood and some sulfadiazine. Fifteen hours later, deep jaundice was noted. In spite of further transfusions and all other therapeutic measures, she did not respond, dying on June 2. She was given, among other things, 10 grams of choline parenterally on May 30 and May 31. The blood culture yielded a pure growth of *E. coli*. On May 29, the cephalin flocculation test was 3 plus and the icterus index 100. Notwithstanding these evidences of liver damage and the abnormal serum lipids, the liver post mortem appeared altogether normal both grossly and microscopically. Acute endometritis and pelvic cellulitis were discovered. It is suspected that the patient had used drugs as well as mechanical means to promote abortion.

A57138, male, born in 1889, had been heavily alcoholic since youth and had been seen in the hospital and emergency ward on various occasions in a state of acute alcoholism. His eating habits were extremely irregular, his diet poor; on one occasion he was suspected of vitamin deficiency. On November 8, 1944, he initiated an alcoholic spree by drinking a pint of rubbing alcohol on a bet. On November 14, he came to the Emergency ward of the New Haven Hospital, weak and tremulous, complaining of a headache and epistaxis. The latter had recurred at intervals for 2 days. On November 16 he was admitted to the hospital after vomiting blood. He had eaten little since the beginning of his drinking bout. He appeared seriously ill and dehydrated, his liver was slightly enlarged, and he had some tenderness in the calves. The next day he became deeply icteric. Vomiting and epistaxis continued at intervals and ecchymoses appeared at the sites of parenteral fluid injections. He lapsed into coma in the course of the day. From the time of admission until his death he passed no urine and only 120 ml. could be obtained by catheter. This contained albumin, casts, and leukocytes. He died November 18 after a convulsion, despite all therapeutic measures, including frequent parenteral administration of large amounts of fluid containing carbohydrate in adequate quantities. The cephalin flocculation test was strongly positive, the icterus index 100, and bromsulfalein retention more than 50 per cent at the end of 30 minutes. The liver was grossly fatty, the pancreas normal, spleen not enlarged. There was extensive degeneration and necrosis of the epithelium of the convoluted tubules of the kidneys.

19694, male, born in 1913, was admitted to the New Haven Hospital on March 5, 1943. For about 8 months he had been exposed in his occupation to an organic halide preparation. He continued to work for a week after the appearance of jaundice, 3 weeks before admission, but was then forced to desist because of weakness and vomiting which increased steadily. On March 2, blood was noted in the vomitus, and on March 4 he became stuporous. On admission he was comatose, deeply jaundiced, and retching. There was a papular rash on his back. The icterus index was greater than 125. He died within 24 hours. At autopsy, the liver was the site of profuse hemorrhages and extensive necrosis, with little evidence of regeneration.

A47621, female, born in 1908, had been treated for syphilis from 1935 to 1937. Both Kahn and Wassermann tests were negative. Enlargement of the liver and spleen were discovered when she was admitted to the hospital in 1939 because of alcoholism and lysol poisoning. She was readmitted in 1942 because of a fissure in ano, external hemorrhoids and a secondary anemia. The last probably arose from rectal bleeding together with malnutrition. The liver was again noted to be slightly enlarged. On January 4, 1943, she again entered the hospital with erysipelas of the left leg. This time she had slight ascites and subcutaneous edema. The lipid studies were made after the erysipelas had subsided. On April 29, she returned to the hospital in shock, almost exsanguinated from a ruptured esophageal varix, and died an hour later despite transfusion and infusions. Autopsy revealed a distinct portal cirrhosis with rather extensive acute necrosis in the central portions of the islands of liver tissue.

B43658, male, born in 1884, was first seen in the Dispensary on March 31, 1943, with a history of edema of the feet and legs for 2 to 3 weeks, with exertional dyspnea, unproductive cough, and scleral icterus, with light-colored stools for a week. He had an enlarged heart, ascites, a large liver, and marked edema of the legs. There was no alcoholic history. The icterus index was 20. He was admitted to the New Haven Hospital May 29, somewhat stuporous, with edema up to his nipples. On May 31, his gums began to bleed, and on June 2 gross rectal bleeding was noted. The next day his temperature rose sharply to 104.6° F. He died June 4. The icterus index was 35, the cephalin flocculation test 2 plus. Autopsy revealed a classical picture of portal cirrhosis with enormous amounts of fibrous tissue and small islands of liver tissue in which the cells appeared relatively normal.

A45460, male, born in 1883, was in the habit of consuming 4 to 5 quarts of beer daily in addition to an occasional glass of whisky. In 1939, he was admitted to the New Haven Hospital because of nausea, vomiting, diarrhea, and jaundice. The liver was greatly enlarged, the icterus index 55. He ran a febrile course for 2 weeks, after which he gradually improved, the liver diminishing in size and jaundice receding. He was again admitted to the New Haven Hospital November 12, 1944, with ascites of 8 weeks' duration, productive cough, exertional dyspnea, and bleeding from the rectum. His abdomen was greatly distended with fluid. After removal of 10 liters, the liver could just be felt beneath the costal margin; the spleen was never palpable. Skin and sclerae were moderately icteric, the icterus index was 50, and bromsulfalein retention 24 per cent at the end of 30 minutes. On November 15, he suddenly vomited bright-red blood, his blood pressure fell progressively, and he lapsed into coma. He died later in the day. His blood sugar shortly before death was 46 mgm. per cent. Post mortem examination revealed an advanced portal cirrhosis of the liver in which the connective tissue and, to a lesser extent, the islands of parenchyma were densely infiltrated with mononuclear cells and with numerous polymorphonuclear leukocytes.

B43742, male, born in 1889. In 1926, during a cholecystectomy, some generalized fibrosis of the liver was

noted. In 1939 or 1940, enlargement of the liver was reported. From November, 1942, he noticed increasing malaise, loss of weight, anorexia and flatulence. In March, 1943, after a coryza, he was seized with stabbing pain in the right axilla, accompanied by a cough and a temperature of 101° F. Five days later, jaundice appeared and increased steadily. Shortly after this, the stools became black from changed blood. He was admitted to another hospital, deeply jaundiced, with a large liver and fever. Because of anorexia, vomiting, and hiccough he was unable to eat and deteriorated rapidly. On March 3, he was transferred to the New Haven Hospital in a stuporous state, extremely emaciated, with tense ascites, and a huge liver. He died on March 6. The icterus index was 60, cephalin flocculation 3 plus, prothrombin time 120 seconds (normal control 12.8). Autopsy revealed diffuse fibrosis of the liver without large scars, some necrosis of the liver cells, some proliferation of the bile ducts, and moderate evidence of biliary stasis. Little sound liver tissue remained. In addition, there was carcinoma of the liver. The sample for analysis was taken from an area free from carcinoma.

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ELECTROPHORETIC CHANGES IN THE PLASMA PROTEIN PATTERNS OF PATIENTS WITH RELAPSING MALARIA¹

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Numerous studies of the serum or plasma proteins in malaria have shown that there is a depression of albumin and a relative rise of globulins following paroxysms. Two workers (1), from a review of the earlier literature and from experimental studies with monkey malaria, concluded that, while the changes were not specific for malaria, the amount of change generally correlated with the intensity of an attack. In the interval between relapses, the serum total protein and albumin:globulin values tended to return toward normal, an improvement that was accelerated by treatment. Later observations on human subjects (2 to 4) have also been in accord with these findings.

Although it has been suggested (5) that the increase in the serum fraction precipitable by 13.5 per cent sodium sulfate might be sufficiently characteristic of the disease to be the basis of a diagnostic test for malaria, the subsequent use of this test (6) indicated that similar increases would also be encountered in a variety of other diseases.

All previous studies have been made with some type of salt fractionation. The purpose of the present study was to supplement these earlier observations by electrophoretic analyses, and to ascertain whether or not any test based upon changes in the plasma protein pattern might be of diagnostic use in the group of patients with relapsing malaria.

PROCEDURE

The 8 subjects studied were admitted because of malarial relapses. All had first been exposed to infection about 12 months prior to admission. In the intervening period they had had a variable number of relapses (2 to 12) but no complications. Their nutritional status was

good owing to the prompt medical attention that they had received for each relapse. Records of earlier attacks indicated that several of the patients at least had originally had mixed infections (*P. vivax* and *P. falciparum*) but at the time of these studies the *falciparum* parasites had apparently disappeared as only *vivax* forms were found in numerous blood smears. While the group is restricted rather than representative of more extreme cases of the disease, the subjects are typical of the otherwise healthy patients in whom it is desired to recognize latent infection prior to a relapse.

Each of the 8 patients was allowed to have 3 paroxysms, which were tolerated without undue discomfort. At the time of the third paroxysm, the course of the disease was interrupted with atabrine or quinine therapy in all but 1 patient (Sch.). This patient spontaneously arrested his paroxysms after the second. On the morning following admission a fasting blood sample was taken for determinations of the plasma protein concentration by the Kjeldahl method, the albumin:globulin ratio by salt fractionation, and for electrophoretic analysis by procedures previously described (7). Subsequent samples were taken at various times as indicated in Table I and in Figure 1.

RESULTS AND DISCUSSION

Although the total protein concentrations remained within normal limits, the albumin:globulin ratios were found to be depressed both in the chemical and in the electrophoretic analyses, thus confirming reports in the literature. It is seen (Table I, Figure 1) that the globulin increase occurs principally in the fibrinogen and the γ globulin fractions. The latter is of interest both because it probably carries antibody activity (8) and because it presumably is the main fraction precipitated from serum by 13.5 per cent sodium sulfate.

The significant fact, however, is that the pattern tends to return to normal despite persistent infection after the paroxysms are interrupted by treatment. Each of the patients of Figure 1 (Sp., Tr.) had a further relapse not due to reinfection with 2 months of discharge from the hospital. The abnormalities in protein pattern thus appear

¹ The Bureau of Medicine and Surgery does not necessarily undertake to endorse views or opinions which are expressed in this paper.

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TABLE I

Plasma protein data from six malarial patients

Days are counted from the first paroxysm of the current relapse. The last 6 columns give the fractions of the total protein furnished by the components: Albumin, α_1 , α_2 , and β globulins, fibrinogen, and γ globulin. Note that the average includes data given graphically in Fig. 1. The normal values are from reference 7.

Patient	Day	Total protein	Albumin:Globulin		Alb. T	α_1 T	α_2 T	β T	δ T	γ T
			Salt fractionation	Electrophoresis						
		<i>grams per 100 ml.</i>								
Sh (No. 11539)	7	6.71	1.44	1.00	0.500	0.052	0.066	0.152	0.079	0.151
	12	6.48	1.17	0.91	0.477	0.062	0.070	0.120	0.096	0.175
	21	6.42	1.67	1.22	0.552	0.042	0.065	0.129	0.063	0.149
Ar (No. 11541)	1	6.67	2.06	1.13	0.530	0.059	0.088	0.144	0.068	0.111
	5	6.73	1.50	0.96	0.490	0.057	0.067	0.152	0.076	0.158
Sch (No. 11534)	6	6.30	1.72	1.07	0.517	0.056	0.067	0.149	0.077	0.134
	27	6.51	2.56	1.27	0.559	0.043	0.107	0.113	0.057	0.121
Mu (No. 11554)	4	—	—	0.81	0.448	0.072	0.145	0.152	0.096	0.087
	11	6.33	1.92	1.13	0.531	0.052	0.111	0.143	0.063	0.100
Ma (No. 11549)	35	6.30	1.24	0.95	0.487	0.048	0.045	0.131	0.063	0.226
Pa (No. 11529)	7	6.40	2.01	1.30	0.566	0.036	0.085	0.133	0.052	0.128
Average (including Sp., Tr.)		6.88	1.73	1.07	0.514	0.051	0.083	0.132	0.074	0.145
Normal values		6.70	2.2	1.53 (± 0.18)	0.603 (± 0.028)	0.046 (± 0.007)	0.072 (± 0.013)	0.121 (± 0.019)	0.051 (± 0.006)	0.110 (± 0.025)

to be due to host reaction to the acute process of a paroxysm rather than to the mere presence of parasites in the body. Moreover, the non-specific nature of the γ globulin rise is attested by the increase of this component after streptococcal infections as described in the following paper.

The average mobilities of plasma protein components were within normal limits in the malarial plasmas (Table II), indicating that there was no detectable amount of protein with unusual mobility.

In more severe forms of malaria, an extreme depression of albumin concentration may occur, as shown by the data of Table III. This patient,³ not included in the averages of Tables I and II,

³ Seen through the courtesy of Dr. Harry Most.

was a woman, age 22, who came under medical observation only after having suffered severe untreated *P. falciparum* malaria for 3 weeks. The apparent increase in mobilities of all components is probably due merely to the greater dilution of proteins.

It appears necessary to conclude that methods based on changes in relative concentrations of the plasma protein components are of no diagnostic value, both because the changes that do occur are non-specific and because the pattern tends to return to normal despite persistent infection. It is, however, probable that determinations of the total protein concentration and of the albumin:globulin ratio would be useful adjuncts in evaluating the severity of the disease.

TABLE II

Mobilities $\times 10^5$ (cm.² per volt second) of the plasma protein components in malarial and normal plasma. Averages and standard deviations are computed from 15 normal (7) and 23 malarial plasmas

	Albumin	α_1	α_2	β	δ	γ
Malaria	5.89 \pm 0.277	4.99 \pm 0.264	4.00 \pm 0.276	3.03 \pm 0.250	2.15 \pm 0.168	1.18 \pm 0.142
Normal	5.94 \pm 0.267	5.07 \pm 0.236	4.08 \pm 0.256	2.83 \pm 0.241	2.14 \pm 0.232	1.02 \pm 0.142

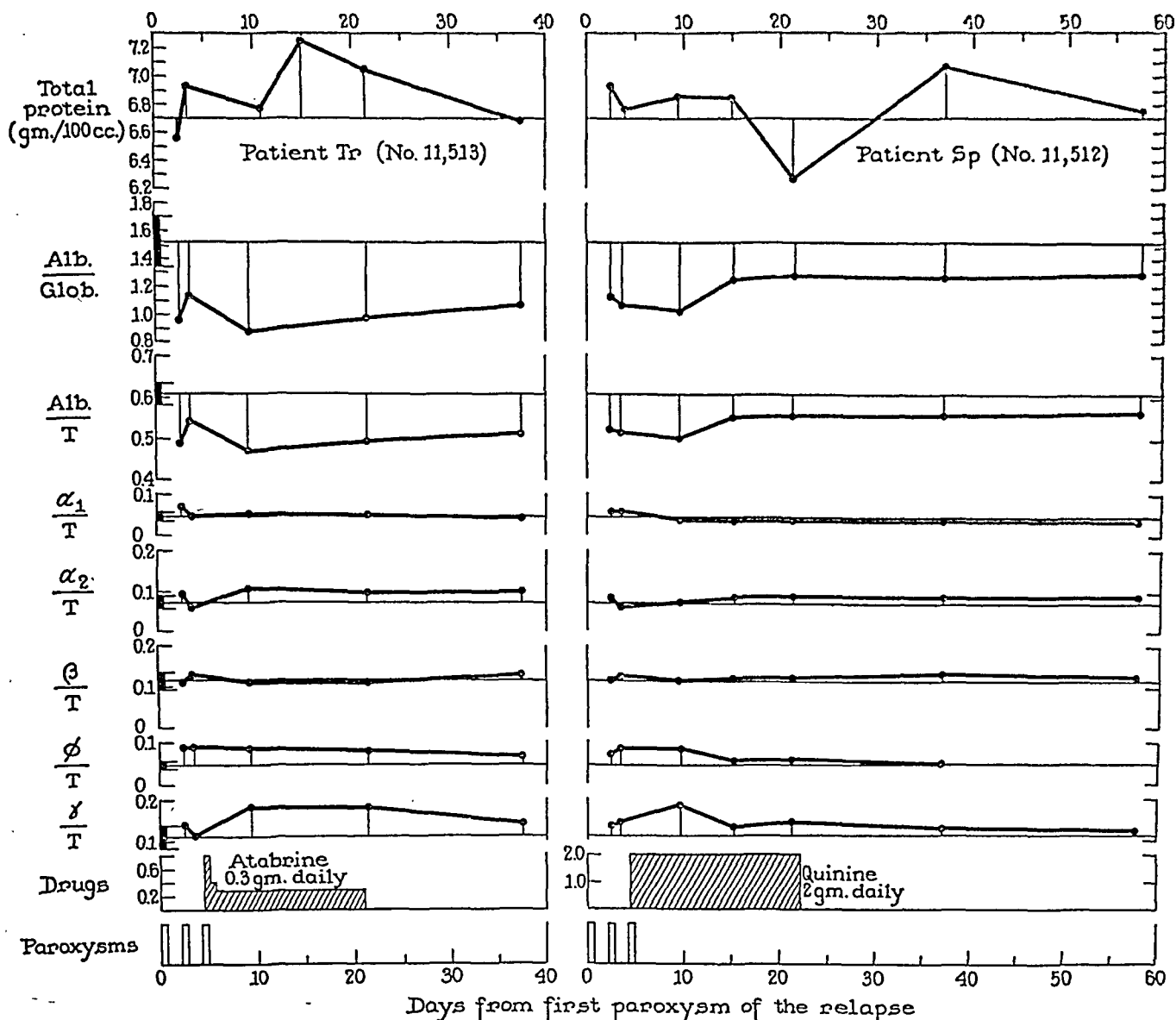


FIG. 1. TIME CHANGES IN THE ELECTROPHORETIC PLASMA PATTERNS OF 2 PATIENTS

Shown on the ordinates are the total protein concentration, the electrophoretic albumin:globulin ratio, and the ratios to total protein concentration (T) of albumin and of the globulins (α_1 , α_2 , β , ϕ , γ), where ϕ denotes fibrinogen. Normal average values for these are given by the light horizontal lines, while normal ranges (of 1 standard deviation) are shown by the heavy brackets at the left. Note that the ordinate scales for drug dosage differ in the 2 cases, although the units (grams per day) are the same.

TABLE III

Data obtained from 1 patient with severe untreated *P. falciparum* malaria

	Total protein	Albumin:Globulin		Alb. T	$\frac{\alpha_1}{T}$	$\frac{\alpha_2}{T}$	$\frac{\beta}{T}$	$\frac{\phi}{T}$	$\frac{\gamma}{T}$
		Salt fractionation	Electrophoresis						
Concentrations	grams per 100 ml. 3.64	1.30	0.72	0.417	0.089	0.086	0.163	0.095	0.147
Mobilities $\times 10^5$ (cm. ² per volt second)				6.34	5.39	4.47	3.18	2.28	1.20

SUMMARY

1. Eight patients with relapsing *P. vivax* malaria were found to have normal total protein concentrations, but depression in albumin and increases in the globulins, notably fibrinogen and γ globulin.

2. In the 2 of these patients that were studied by repeated electrophoretic analyses, the plasma patterns tended during subsequent weeks to return to normal, despite persistent infection as proven by later relapses.

3. The 1 patient with severe *P. falciparum* malaria was found to have an extreme depression of total protein concentration, due mainly to reduction in albumin.

4. It is concluded that the changes in the plasma protein pattern are of no diagnostic value, but that measurement of the total protein concentration and the albumin:globulin ratio may be of aid in evaluating the severity of the disease.

The authors are indebted to Drs. T. Shedlovsky and D. A. MacInnes for their generosity in making available the electrophoretic equipment used in this study.

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ELECTROPHORETIC CHANGES IN THE SERUM PROTEIN PATTERNS OF PATIENTS WITH SCARLET FEVER AND RHEUMATIC FEVER¹

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It is generally recognized that rheumatic fever is usually, if not always, preceded by a group A streptococcal infection. On the other hand, only a few of those individuals who suffer streptococcal infections develop clinical attacks of rheumatic fever. This fact makes it probable that the development of rheumatic fever also involves some peculiarity in the host reaction to the infection. Although immunological changes have been shown to occur in the sera of patients with streptococcal infections and rheumatic fever (1 to 8), there has been no systematic study of the alterations in the overall pattern of serum proteins under these conditions. Since the changes in the serum protein pattern furnish additional data for a description of the host reaction, it seemed desirable to follow these changes in individuals who developed rheumatic fever following scarlet fever and in a control group of patients who had scarlet fever but did not develop rheumatic fever.

MATERIAL AND METHODS

Sera were collected at weekly intervals throughout the hospital stay of 110 patients originally admitted with scarlet fever. These sera were stored in sterile tubes at 4° C. without preservative. Also at weekly intervals, the white blood cell count, erythrocyte sedimentation rate, and serum antistreptolysin O titer were determined; an electrocardiogram was taken, and cultures were made from the nose and throat. Six subjects were selected for electrophoretic study. This group, limited in size by the time required for analyses, was chosen to provide clinically typical examples of rheumatic fever following scarlet fever with comparable controls. None of the patients had previously suffered rheumatic fever or chorea.

The electrophoretic analyses were made in diethylbarbituric acid buffer ($\mu = 0.1$, pH = 8.6) (9). Normal plasma standards, obtained by this method (10), were

adjusted to a serum basis for the present study on the assumption that the loss of fibrinogen removed 5 per cent of the total protein. As a result of storage of sera, some specimens were as old as 18 months when analyzed. It is assumed that aging of the sera did not cause electrophoretically detectable changes except a slight reduction of β globulin due to dissociation of lipid. Probably for this reason the usual β globulin disturbance in the descending channel was absent in aged sera, although present in fresh specimens. The use of aged sera for a quantitative study cannot be justified by direct evidence, but it appeared quite definite that observed changes correlated with the stage of the disease rather than merely with the age of the serum sample.

The erythrocyte sedimentation rate (ESR) was determined by the Westergren method (11), using 3.8 per cent sodium citrate as anticoagulant.

The serum antistreptolysin O titer (ASO) was measured by the dilution technique of Todd (12) as modified by Hodge and Swift (13).

Nose and throat cultures were made in duplicate on fresh rabbit and sheep blood agar plates. The hemolytic streptococci were grouped and typed by the precipitin technique (14).

Sera from 3 different patients were absorbed with 18-hour cultures of streptococci in attempts to elucidate the nature of γ globulin rise. The strains had been preserved in the dried state, according to the technique of Swift (15). In each absorption, a mixture of 2 parts of serum with 1 part of packed, living bacterial cells (washed once with sterile saline) was incubated 30 minutes at 37° C. Electrophoretic analyses were then made on absorbed serum, cleared by centrifugation, and on an unabsorbed control portion.

Subjects of the study

Rheumatic Fever (Figure 1), J. K. (No. 11371), a 25-year-old male, was admitted on the 4th day of illness with moderately severe scarlet fever due to group A, type 19 hemolytic streptococcus. From the 7th to 18th day of his illness, he was given sulfadiazine in treatment of a marked purulent nasal discharge and continued low grade fever. While under this treatment he became asymptomatic and afebrile. On the 22nd day of his illness, however, he developed anorexia, and general malaise, followed in several days by fever, and migratory joint pains with a secondary rise in the erythrocyte sedimentation rate and first degree A-V heart block. These

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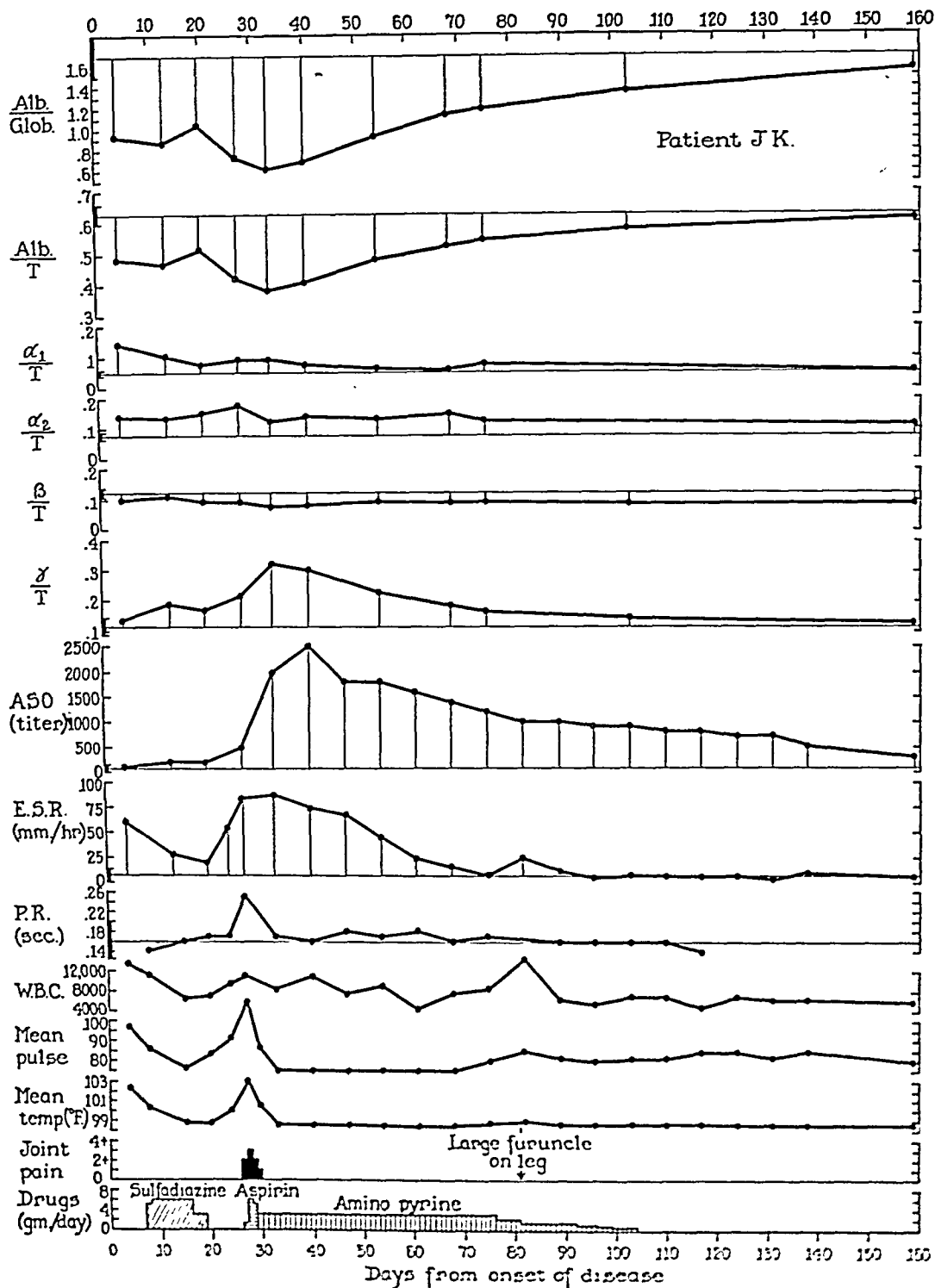


FIG. 1. RHEUMATIC FEVER

From the top downwards, the ordinates indicate the albumin:globulin ratio ($\frac{\text{Alb.}}{\text{Glob.}}$), the concentrations of serum protein components relative to the total protein (T) as measured from the areas of the electrophoretic patterns, the titer of antistreptolysin O (ASO), the erythrocyte sedimentation rate (E.S.R.), the electrocardiographic P-R interval (P.R.), the white blood cell count (W.B.C.), and clinical data. Horizontal lines show normal average values, with normal ranges indicated by brackets at the left.

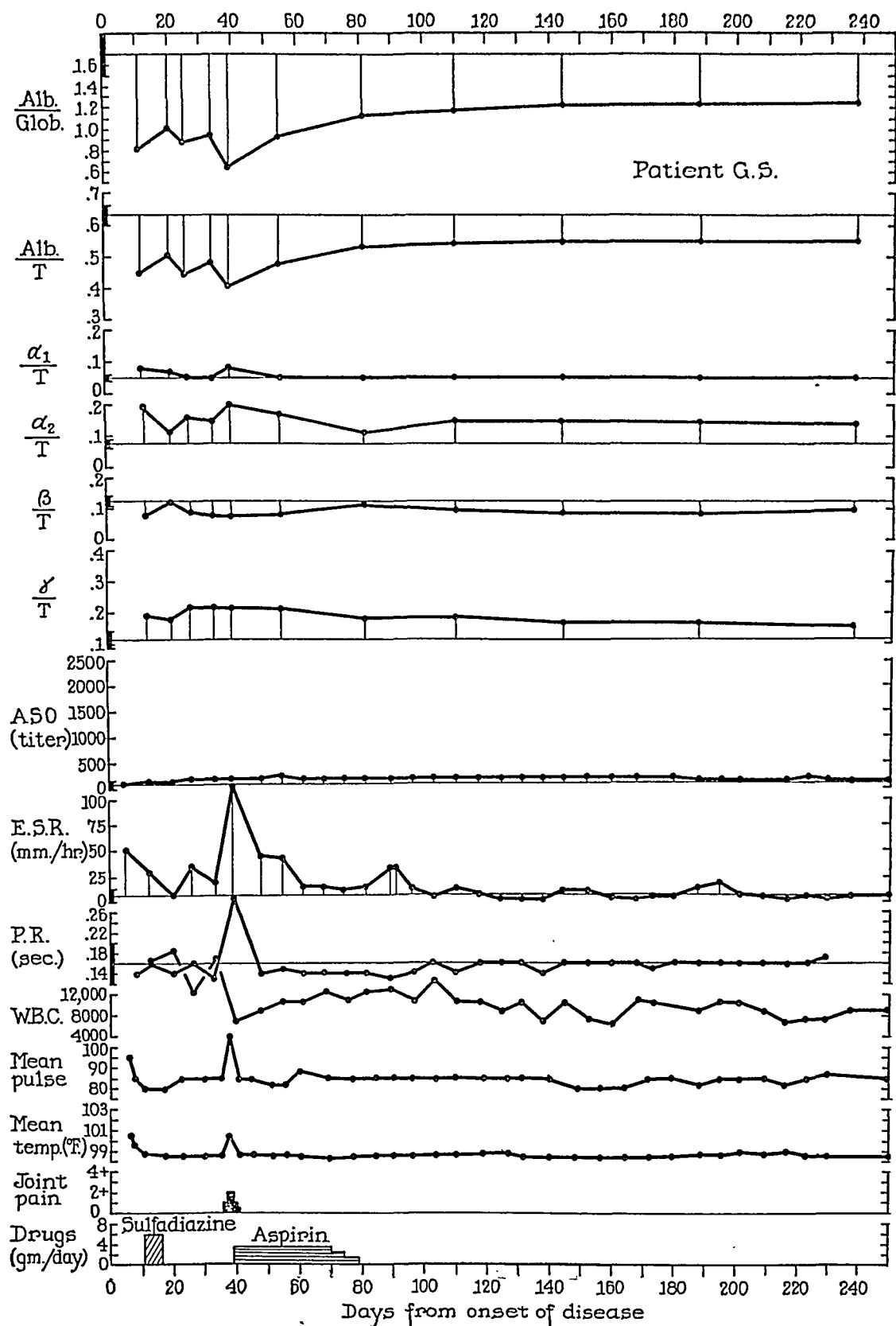


FIG. 2. RHEUMATIC FEVER
Symbols as in Figure 1.

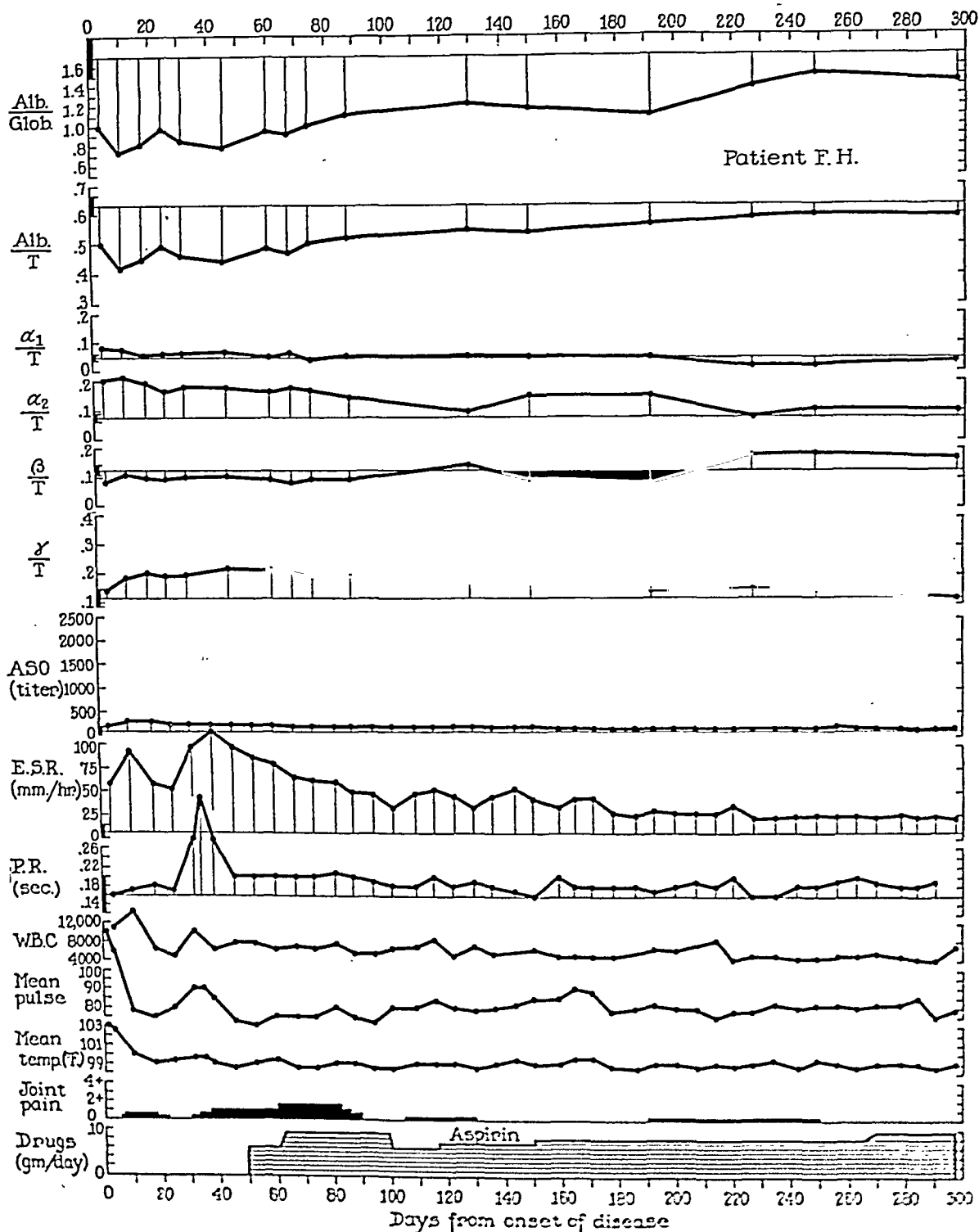


FIG. 3. RHEUMATIC FEVER WITH A PROTRACTED COURSE

Symbols as in Figure 1.

symptoms abated under treatment with aspirin, for which aminopyrine was later substituted because of abdominal discomfort. Convalescence was thereafter uneventful except for a transient disturbance due to a furuncle on the leg. This patient was selected for study because he developed a typical attack of rheumatic fever with a single cycle of activity and a relatively high antistreptolysin O titer following scarlet fever.

Bacteriology. Nose and throat cultures, done weekly, showed the persistence of type 19 streptococci from admission to the 106th day. In addition, two other group A strains were recovered: a type 6 on the 7th day and another strain, not belonging to one of the known types, on the 19th day. The type 19 strain was presumed to have been the cause of the scarlet fever since the patient contracted his disease during an epidemic due to this type, which was also recovered in pure culture on admission. The unidentified type persisted throughout the hospital stay.

Rheumatic Fever (Figure 2), G. S. (No. 11328), a 32-year-old male, was admitted on the 5th day of a mild scarlet fever attack due to group A, type 19 hemolytic streptococcus. Within 3 days he became asymptomatic. From the 12th to 17th day of illness he received sulfadiazine in an unsuccessful attempt to clear his carrier state. On the 20th day he was asymptomatic with a normal erythrocyte sedimentation rate, but he had a leukocytosis of 20,000, and showed in the electrocardiogram diphasic T-waves in leads II and III with low amplitude of the T-waves in the limb leads. On the 35th day, he developed pains in the left shoulder and both wrists, with fever, increased pulse, partial A-V heart block, and a secondary rise in the erythrocyte sedimentation rate. Involvement of other joints occurred to the 38th day when he was started on salicylates with prompt clinical response. During the month following withdrawal of this medication on the 78th day, the erythrocyte sedimentation rate became elevated but he remained asymptomatic. Through the subsequent 4 months he remained asymptomatic with a normal sedimentation rate, but continued to have slight leukocytosis, and changes in the form and direction of the T-waves in leads I and II of the electrocardiogram. During the final month, these abnormalities were absent. At no time was there evidence of valvular disease. This patient was selected for study as a typical rheumatic fever subject. In contrast to the history of patient J. K., the onset of his rheumatic fever was more insidious, the course was more protracted, and the rise of antistreptolysin O titer was only slight.

Bacteriology. The type 19 streptococcus was found in weekly nasopharyngeal cultures throughout his entire hospital stay.

Rheumatic Fever (Figure 3), F. H. (No. 11574), a 23-year-old female, was admitted on the 2nd day of her illness with severe scarlet fever due to group A, type 17 hemolytic streptococcus. During the next 4 days she continued to appear toxic, then gradually improved but did not become afebrile until after the 15th day of illness. The appearance of pain in the cervical spine on the 5th day of illness was followed by variable, low-grade pains

in the knees, left wrist, and left shoulder, during the ensuing 3 weeks. A typical attack of rheumatic fever developed about the 30th day with fever, increased pulse rate, leukocytosis, partial A-V heart block, secondary rise in the erythrocyte sedimentation rate, and recurrent joint symptoms. At this time, there was a systolic murmur at the apex. On the 50th day, she was started on aspirin therapy, which was continued over the following 8 months. Despite the medication she continued to have intermittent joint pains until the last month. During the 4th month, an aortic diastolic blow was first noted; at this time a subcutaneous nodule was found over the right patella. At the time of discharge to another hospital on the 299th day, she was asymptomatic on 9.5 grams of aspirin daily. The heart was of normal size with an aortic diastolic blow. She was selected for study as an example of rheumatic fever with a protracted course.

Bacteriology. The type 17 streptococcus was found in nasopharyngeal cultures throughout the entire hospital stay.

Uncomplicated Scarlet Fever (Figure 4a), W. K. (No. 11341), a 19-year-old male, was admitted on the 3rd day of illness with fairly severe scarlet fever due to group A, type 19 hemolytic streptococcus. He became asymptomatic by the 6th day of illness, but was started on sulfadiazine at this time in an unsuccessful attempt to clear his carrier state. After an uneventful convalescence, he was discharged on the 48th day. This patient represents a typical severe scarlet fever patient with an uneventful convalescence.

Bacteriology. The original type of streptococcus was found in weekly cultures of the nose and throat throughout the period of observation.

Uncomplicated Scarlet Fever (Figure 4b), J. J. (No. 11291), a 20-year-old male, was admitted on the 3rd day of illness with severe scarlet fever due to group A, type 19 hemolytic streptococcus. A few days after admission he became asymptomatic but was started on sulfadiazine, followed by nose and throat sprays of Zephiran and tyrothricin in unsuccessful attempts to clear his streptococcal carrier state. After an uneventful convalescence, he was discharged on the 77th day. This patient, like W. K., was representative of patients with uncomplicated scarlet fever.

Bacteriology. The original type of streptococcus was found in weekly cultures of the nose and throat throughout the period of hospitalization.

Scarlet Fever Complicated with Purulent Sinusitis (Figure 4c), D. S. (No. 11312), a 23-year-old male, was admitted on the 3rd day of illness with severe scarlet fever due to group A, type 19 hemolytic streptococcus. On symptomatic treatment only, he showed gradual improvement until the 13th day, when he first complained of pain over the right side of the face. Examination revealed tenderness and edema over the right antrum; x-rays on the following day showed clouding of the right maxillary and ethmoid sinuses. Within 48 hours after the institution of sulfadiazine therapy and saline irrigations of the right antrum, he became afebrile and asymptomatic. He remained so thereafter until discharge on

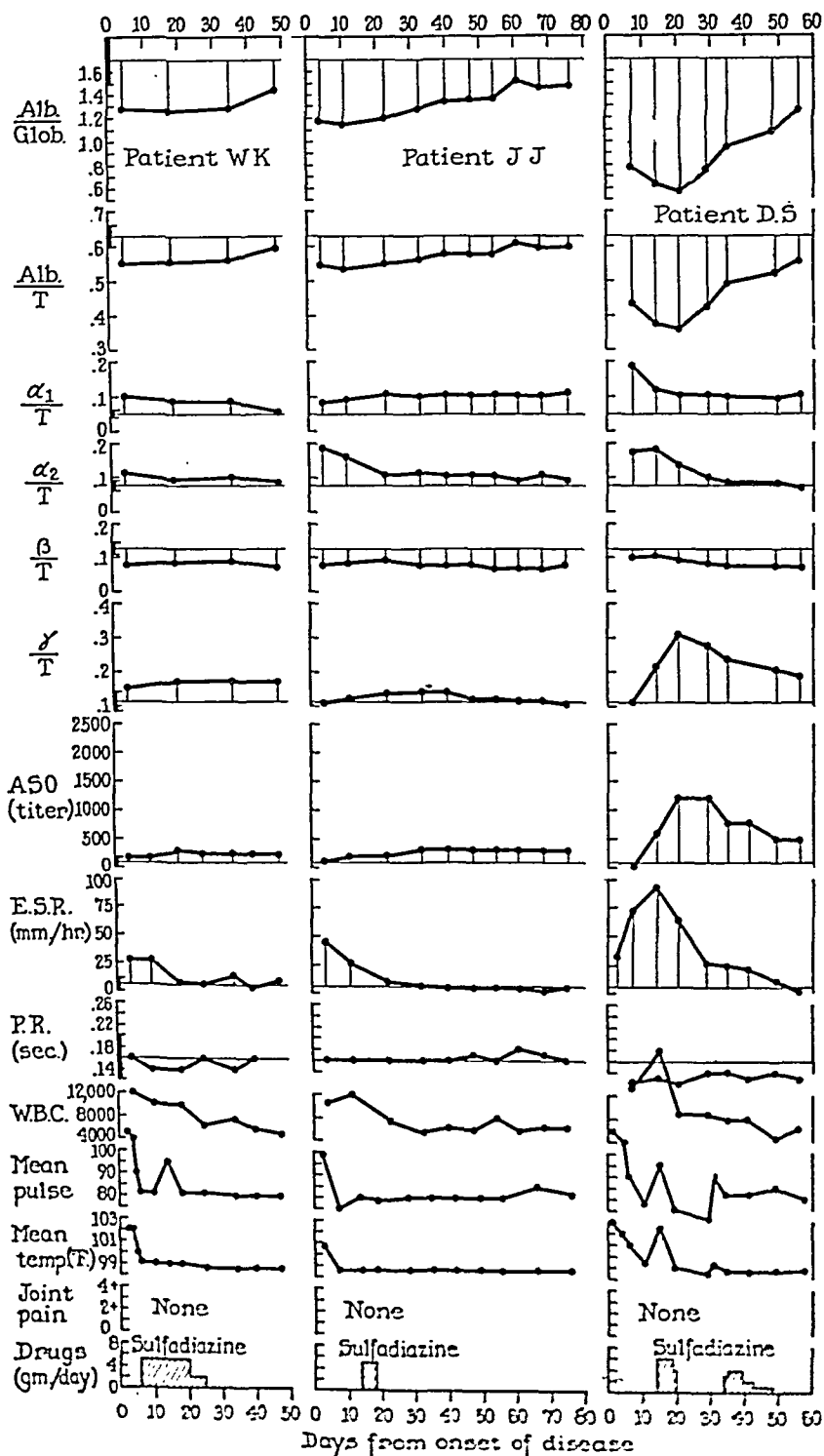


FIG. 4. SCARLET FEVER WITHOUT RHEUMATIC FEVER

Patients W. K. and J. J. were uncomplicated; patient D. S. had purulent sinusitis as a complication. Symbols as in Figure 1.

the 59th day. The patient was selected for study to illustrate the effect of a purulent complication of scarlet fever in a non-rheumatic subject.

Bacteriology. The original type of streptococcus was found in weekly nose and throat cultures to the 34th day and in the purulent sinus exudate on the 15th day. No hemolytic streptococci were found in nose and throat cultures after the 34th day.

OBSERVATIONS AND DISCUSSION

The electrophoretic changes in the serum protein patterns of 2 patients with uncomplicated scarlet fever, one patient with a purulent complication, and 3 who developed typical attacks of rheumatic fever, are illustrated in Figures 1 through 4. Marked, systematic changes from the normal serum protein pattern (10) were found in all cases. The relative concentration of albumin was depressed by the attack of scarlet fever, with a consequent marked reduction in the albumin-globulin ratio. The slow rise toward normal in convalescence from scarlet fever was apparently delayed by the development of clinical rheumatic fever. Of the globulins, α_1 was elevated with the scarlet fever attack to a relative concentration 2 to 3 times the normal value, and fell toward normal as rapidly in rheumatics as in non-rheumatics. The α_2 globulin, likewise elevated with the scarlet fever, tended to maintain an abnormal elevation in the rheumatic patients, in accord with the finding of another group of workers (16). The β globulin showed no characteristic change.

Of particular interest are the changes in the relative concentration of γ globulin since some antibodies, at least, are contained in this fraction of the serum (17). Indirect support of the pre-

sumption that specific antibodies induced by the streptococcal infection also move with the γ globulin is found in the general correlation between the titer of antistreptolysin O and the relative concentration of γ globulin (Figures 1 to 4). It should not be assumed, however, that the rise of γ globulin was quantitatively due to increase of this antibody alone, since in all probability there were increased amounts of other antibodies, both specific for the infection and anamnestic. An estimation of the amount of antistreptolysin O present could not be made for want of a suitable technique for absorption with the streptolysin O.

Absorption experiments were done on the sera of 3 patients to determine whether or not an appreciable part of the γ globulin rise might be due to antibodies directed toward the somatic antigens of the streptococcal cell. In each of the 3 cases, the serum was absorbed with the living streptococcal cells of the strain that had induced the patient's attack of scarlet fever. There was sufficient serum from only 1 of the patients to include a heterologous-type control. As may be seen in Table I, no significant changes were found in the relative concentrations of γ globulin or of the other protein components following these absorptions. A decrease of at least 10 per cent in the concentration of γ globulin would have had to occur to be significant. The slight decreases in the titer of antistreptolysin O are about at the limit of experimental error. If not fortuitous, they are probably due to small amounts of streptolysin introduced with the living streptococci.

Whatever may be the explanation of the rises in γ globulin, it is clear that these increases in

TABLE I

Absorptions of sera with streptococci

The first 5 rows indicate the fraction of the total serum protein (T) furnished by the serum protein components, as estimated from the areas of the electrophoretic patterns. ASO is antistreptolysin O titer

	Patient A. W.†			Patient D. S.		Patient J. K.	
	Unabsorbed control	Absorbed with patient's strain	Absorbed with heterologous type	Unabsorbed control	Absorbed with patient's strain	Unabsorbed control	Absorbed with patient's strain
Alb./T	0.436	0.432	0.402	0.448	0.447	0.482	0.508
α_1 /T	0.091	0.108	0.105	0.102	0.095	0.046	0.087
α_2 /T	0.193	0.193	0.214	0.106	0.102	0.155	0.094
β /T	0.090	0.099	0.095	0.089	0.093	0.094	0.075
γ /T	0.190	0.178	0.184	0.264	0.262	0.232	0.237
ASO	600	500	500	1000	800		

† This patient, not otherwise included in the study, had a typical acute attack of rheumatic fever following a group A, type 5 streptococcal pharyngitis.

relative concentration were not correlated with rheumatic fever as such, since comparable increases in this fraction were observed in the non-rheumatic who had a purulent sinusitis (D. S.), and in a rheumatic (J. K.), while slight to moderate increases only were found in 2 non-rheumatics (W. K., J. J.) and in 2 rheumatics (G. S., F. H.).

The mobilities of the globulin components, relative to the albumin mobility, are given in Table II. These values are not precise, owing in part

TABLE II

The mean relative mobilities of the globulin components relative to albumin
(Normal standards are calculated from data in reference 10)

	α_1	α_2	β	γ
Normal	0.854	0.687	0.476	0.172
Rheumatic	0.861	0.674	0.509	0.197
Scarlet fever, non-rheumatic	0.851	0.679	0.517	0.213

to the variations in protein concentration in the samples analyzed. It is also possible that variations in the relative mobilities resulted from aging of the sera, although no correlation could be found between change in relative mobilities and age of sera at the time of analysis. It appears, despite these uncertainties, that there were no significant differences in relative mobilities to distinguish the sera of the 3 rheumatic subjects from those of the 3 non-rheumatic scarlet fever controls.

The serum protein changes in the non-rheumatic patients presumably reflected a host reaction to infection with hemolytic streptococci. In the rheumatic subjects, although the original changes can be explained equally well by the same host reaction, the reason for the great delay in return to normal is not evident. Persistence of the micro-organism in the nasopharynx was found in the non-rheumatic, as well as in the rheumatic subjects. In view of the non-specific nature of the observed serum protein changes, it can only be said that the protracted changes reflected the physiological disturbances associated with continued rheumatic inflammation. Except, therefore, for the greater duration of abnormalities in these patients, there appeared to be no electrophoretic changes that distinguished the sera of patients who developed rheumatic fever after scarlet fever from the sera of patients who had scarlet fever alone.

SUMMARY AND CONCLUSIONS

1. The changes in the serum protein pattern were followed by electrophoretic analyses in 6 patients originally admitted with scarlet fever, 3 of whom subsequently developed rheumatic fever.

2. Systematic, marked changes of a qualitatively similar nature were found in both rheumatic and non-rheumatic subjects; an early depression of albumin with rises in the α_1 and α_2 globulins, and a delayed rise in γ globulin. These abnormalities were, however, more prolonged in the rheumatic patients.

3. A general correlation was found between the increase in γ globulin and the titer of antistreptolysin O. In 3 cases tested, no detectable amount of γ globulin could be removed by absorption with the living streptococcal cells of the strain that had induced the patient's attack of scarlet fever.

4. No significant changes were found in the relative mobilities of the globulin components either in the rheumatic or non-rheumatic group.

5. It was concluded that there were no changes in the serum protein pattern, detectable by the method used, to distinguish rheumatic from non-rheumatic patients. The greater duration of abnormalities in the rheumatics was assumed to be a reflection of the physiological disturbances associated with continued rheumatic inflammation.

The authors are indebted to Drs. T. Shedlovsky and D. A. MacInnes for their generosity in making available the electrophoretic equipment used in this study.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION. XXIV. STUDIES ON THE NUTRITIVE VALUE OF HUMAN PLASMA FRACTIONS¹

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(Received for publication February 5, 1945)

Dried human blood plasma was previously found (1) to be a protein of very poor nutritive value as measured by its ability to support growth in young rats when it served as the only source of protein. Isoleucine was shown to be the chief amino acid deficiency under these conditions. These results with human plasma and young growing rats may be contrasted with the results obtained by some workers (2) and by others using bovine plasma and hypoproteinemic dogs. They found that bovine serum proteins serve well for the regeneration of serum proteins in such dogs. Whether these differences with respect to the nutritional qualities of the plasma proteins represent (a) differences in the requirement of the 2 species, (b) differences in the requirement of animals of different ages, (c) differences in the requirement for growth as compared to serum regeneration, or (d) a variation in the amino acid composition of the proteins used is as yet unknown.

The nutritive value of plasma or of the various plasma proteins may be of importance since certain individuals may receive them as the sole source of protein at a time when protein requirements are especially high. Albumin is the protein most abundant in plasma and the protein which may be used in relatively large amounts in intravenous therapy. It was of interest, therefore, to determine the nutritive value of this protein as well as the other plasma proteins available in sufficient amounts for study and possible therapeutic use. In addition to the rat growth studies, we report observations on the nutritive value of

human albumin as measured by another technique, namely, nitrogen balance in the adult dog.

EXPERIMENTAL

Rat studies. As in the previous work with plasma using young growing rats (1), the plasma fractions replaced sucrose in the purified basal ration composed of salt mixture 4 per cent, corn oil 4 per cent, and sucrose to 92 per cent. Sufficient amounts of the protein supplements including amino acids, if these were added, were used to give a protein content ($N \times 6.25$) of 20 per cent. Thiamine chloride, riboflavin, pyridoxine hydrochloride, calcium pantothenate, nicotinic acid, and choline chloride were added in excess of requirements as the crystalline compounds, and haliver oil fortified with viosterol was fed by dropper twice weekly.

In Experiment I, groups consisting of 3 rats each, average weight 52 grams, were fed rations in which the only source of protein was gamma-globulin, fibrin or albumin

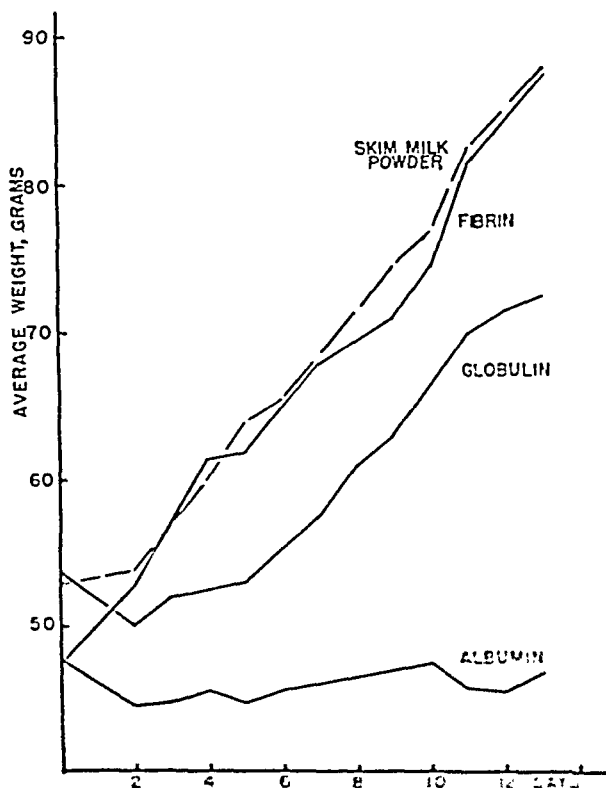


FIG. 1. GROWTH CURVES OF RATS REceiving VARIOUS PROTEINS

¹ The products of plasma fractionation employed in this work were developed by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

This paper is Number 34 in the series "Studies on Plasma Protein" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

from human plasma. Skim milk powder was the protein used for the control animals. The growth curves are shown in Figure 1. Albumin supported no growth at all over this period while fibrin compared favorably with skim milk powder and globulin was intermediate between these 2 extremes. As the previous experiments with whole plasma had shown it to be a relatively poor protein (1), it was evident that the amounts of fibrin and globulin present in plasma are too low to supplement albumin adequately.

Since isoleucine is the limiting amino acid in plasma, it was assumed that it was also limiting in albumin. However, the composition of the plasma proteins as given by another group of workers (3) shows a very low content of tryptophane for albumin. These 2 amino acids were investigated in Experiment II, the growth curves of which are shown in Figure 2. Four groups of young rats were fed rations containing 20 per cent albumin with the following additions: Group 1, none; Group 2, 0.3 per cent *l*(-)-tryptophane; Group 3, 1.2 per cent *dl*-isoleucine; and Group 4, 0.3 per cent *l*(-)-tryptophane and 1.2 per cent *dl*-isoleucine. Group 4 was discontinued after a few days when it became apparent that growth was rapid upon this ration. The other 3 groups were continued upon their respective rations until the tenth day when both amino acids were added to each ration. The growth response was immediate in all cases showing that albumin is so low in both of these amino acids as to completely prevent growth. Isoleucine and tryptophane are certainly the chief amino acid deficiencies for the growing rat when albumin is the sole source of protein.

The approximate amount of each of these amino acids that is necessary to give optimum growth in young rats was determined in Experiments III and IV. In Experi-

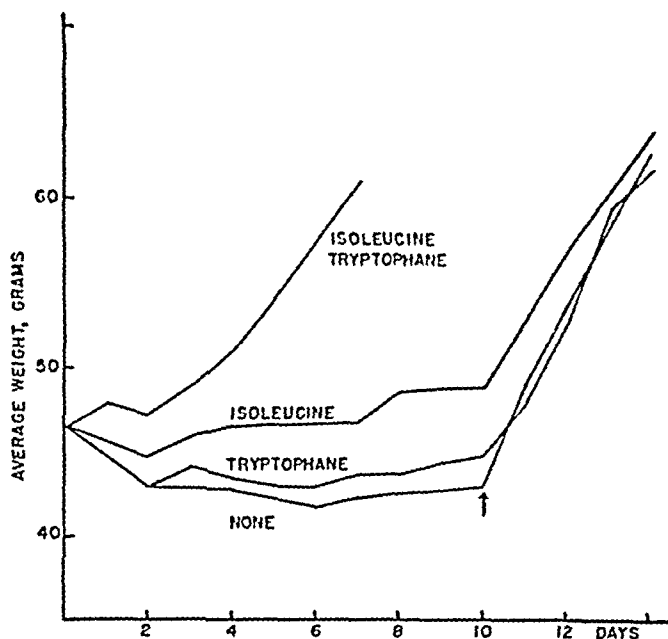


FIG. 2. THE EFFECT OF ISOLEUCINE AND TRYPTOPHANE SUPPLEMENTS ON THE GROWTH OF RATS RECEIVING ALBUMIN AS THE SOLE SOURCE OF PROTEIN (BOTH AMINO ACIDS ADDED ON THE TENTH DAY)

ment III, 28 rats weighing approximately 60 grams each were equally divided into 7 groups. Each group received the basal ration containing 1 per cent *dl*-isoleucine and sufficient albumin to give a total of 20 per cent protein ($N \times 6.25$) in the ration. *l*(-)-Tryptophane was added at the following levels: 0.0, 0.01, 0.02, 0.03, 0.05, and 0.075 per cent of the ration. In Figure 3, the gain in weight of these groups during a 15-day period is plotted against the

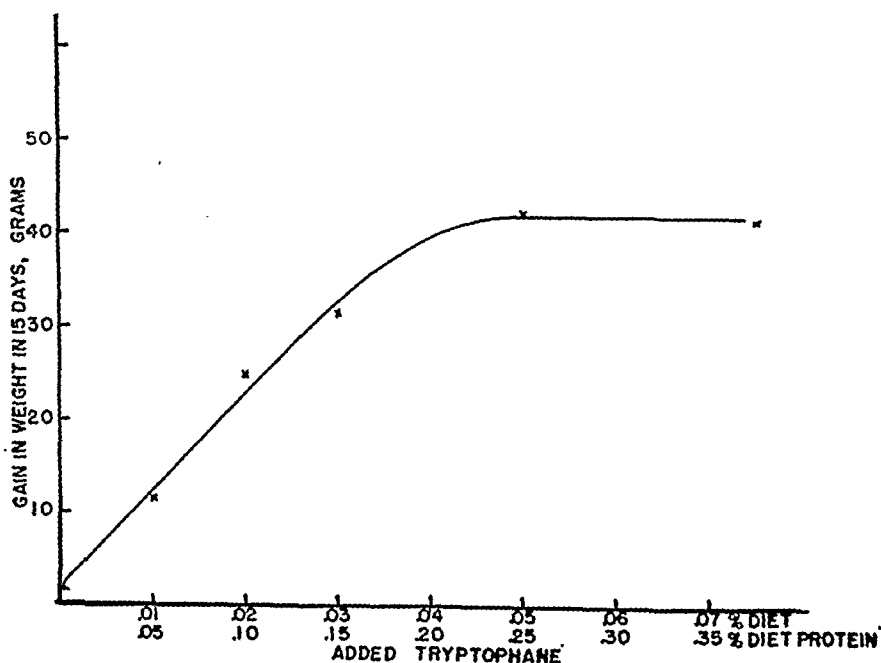


FIG. 3. GAIN IN WEIGHT ON VARIOUS LEVELS OF TRYPTOPHANE ADDED TO ALBUMIN SUPPLEMENTED WITH 1 PER CENT *dl*-ISOLEUCINE

TABLE I
Rat growth in relation to tryptophane intake and protein efficiency

Group	Albumin in diet*	l(-)-Tryptophane added		Total tryptophane content†		Protein consumed per rat per day	Tryptophane consumed per rat per day	Gain in weight per rat per day	Protein‡ efficiency
		To diet	To dietary protein	Diet	Dietary protein				
	per cent	per cent	per cent	per cent	per cent	grams	mgm.	grams	
1	19.34	0.0	0.0	0.035	0.175	1.00	1.75	0.116	0.12
2	19.34	0.01	0.05	0.045	0.225	1.44	3.24	1.44	1.00
3	19.34	0.02	0.10	0.055	0.275	1.49	4.10	1.67	1.12
4	19.34	0.03	0.15	0.065	0.325	1.53	4.97	2.07	1.35
5	19.34	0.05	0.25	0.085	0.425	1.81	7.70	2.81	1.55
6	19.34	0.075	0.375	0.110	0.550	1.72	9.40	2.74	1.59

* One per cent isoleucine supplies nitrogen equivalent to 0.66 per cent protein. This amount was added to all diets.

† Calculated from the tryptophane content of albumin as determined by Dr. Brand.

‡ Grams—gain per gram of protein eaten.

level of tryptophane added to the ration. Maximum growth was obtained at approximately 0.05 per cent of added l(-)-tryptophane. Since this albumin sample contained 0.18 per cent of tryptophane,² the albumin in the ration would add 0.035 of tryptophane to the diets. The diet, which appears optimum, thus contained a total of 0.085 per cent l(-)-tryptophane. According to these results, a protein, when fed at a 20 per cent level, would have to contain at least 0.42 per cent l(-)-tryptophane in order to support good growth in the young rat.³ The data on food intake and the calculations shown in Table I indicate a requirement of about 8 mgm. of tryptophane per day for these rats which had an average weight during

the experiment of 85 grams. The animals received about 94 mgm. per kgm. of body weight per day. In our experience comparable animals of this strain gain from 3 to 4 grams per day on purified rations containing 18 per cent casein. Thus the maximum growth obtained in this experiment was somewhat less than might have been expected and considerably less than obtained in the next experiment discussed below. It is possible that the optimum level of tryptophane may be somewhat higher for groups of animals which show better growth.

The data on a similar experiment in which tryptophane was added in constant amount, in excess of the requirement found in the previous experiment, and the level of dl-isoleucine was varied are shown in Figure 4 and Table II. Maximum growth was obtained when approximately 0.9 per cent of dl-isoleucine was added to the diet. The isoleucine content of the albumin was determined by microbiological methods. The results ranged from 1.5 to 1.8 per cent isoleucine, average 1.7 per cent. Some of the assays were made using the original media of Hegsted (5), and others with a medium in which the amounts of the amino acids in the basal medium were raised to approximate those used by other investigators (6). This change did not affect the results obtained. The calcula-

TABLE II
Rat growth in relation to isoleucine intake and protein efficiency

Group	Albumin in diet*	l(+)-Isoleucine added†		Total l(+)-isoleucine content‡		Protein consumed per rat per day	Isoleucine consumed per rat per day	Gain in weight per rat per day	Protein efficiency
		To diet	To dietary protein	Diet	Dietary protein				
	per cent	per cent	per cent	per cent	per cent	grams	mgm.	grams	
1	20.0	0.0	0.0	0.34	1.70	0.93	15.8	0.0	0.0
2	19.6	0.25	1.25	0.58	2.90	1.51	43.8	2.68	1.77
3	19.5	0.35	1.75	0.68	3.40	1.63	55.4	3.23	1.98
4	19.4	0.45	2.25	0.78	3.90	1.77	69.0	4.09	2.26
5	19.3	0.55	2.75	0.88	4.40	1.75	77.0	4.18	2.39

* All diets were supplemented with 0.25 per cent dl-tryptophane.

† Added as dl-isoleucine.

‡ Calculated on basis of isoleucine content of albumin as 1.70 per cent.

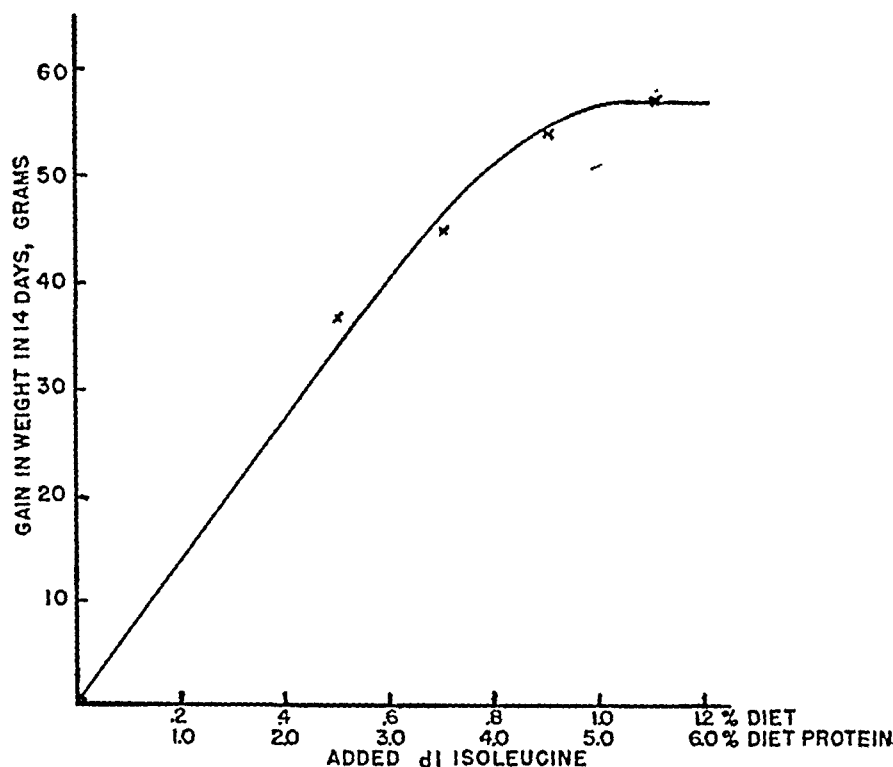


FIG. 4. GAIN IN WEIGHT ON VARIOUS LEVELS OF ISOLEUCINE ADDED TO ALBUMIN SUPPLEMENTED WITH 0.25 PER CENT *dl*-TRYPTOPHANE

tions in Table II are based upon the average figure of 1.70 per cent isoleucine in albumin and on the assumption that only the natural isomer in *dl*-isoleucine is active (7). The isoleucine content of the ration producing maximum growth thus appears to be 0.78 per cent which corresponds to an isoleucine content of the dietary protein of 3.9 per cent. The isoleucine intake of these animals was about 70 mgm. per day, and their average weight during the experiment was 80 grams. On a body weight basis, the intake is thus about 8 or 10 times the tryptophane requirement, approximately 880 mgm. per kgm. per day. In this and in the tryptophane experiment, calculations made at various intervals during the experiment, when both food intake and weight were somewhat less, do not change these results.

Nitrogen balance studies with the dog. The details of this study will be reported later in connection with other similar studies. We have determined the minimum amount of protein required to maintain nitrogen balance in the adult dog fed a purified ration similar to that used in the rat growth experiments, although somewhat higher in fat. With albumin as a sole source of nitrogen, approximately 0.14 gram of nitrogen per kgm. per day was required to maintain positive balance. This corresponds to a protein requirement of 0.875 gram per kgm. per day. If one assumes that tryptophane and isoleucine are the limiting amino acids for the dog, as they definitely are for the growing rat, then the tryptophane requirement would be about 1.6 mgm. per kgm. per day, and the isoleucine requirement about 15 mgm. per kgm. per day. These figures represent studies made over 6-day periods on each level of albumin studied, previous to which the

animal had received the "nitrogen-free" basal ration for 10 days.

DISCUSSION

The striking difference obtained with the growing rat and the adult dog when albumin is the sole source of nitrogen is worthy of consideration. On a caloric basis, it was found that when 19 per cent of the calories were derived from albumin by the young rat, the protein was completely inadequate for growth, while approximately 6 per cent of the caloric intake of the adult dog from this source sufficed for maintenance of nitrogen balance. It is well known that other proteins of high biological value will prove adequate for either species when fed at these respective levels. Calculations upon the tryptophane and isoleucine requirements on the body weight basis for these 2 species further emphasize the great difference in requirement. It is realized that such differences may be due to the physiologic state, *i.e.*, growth *versus* maintenance, but until there is adequate evidence, one should not apply data obtained with 1 animal to others, including man.

It is thus readily apparent that before significance can be assigned to the low tryptophane and isoleucine content of albumin with regard to intra-

venous feeding of man, direct studies upon the human must be obtained. Studies are also needed to determine whether the reestablishment of nitrogen equilibrium and the restoration of the losses of body nitrogen after injury demand protein supplies similar to those of growth or of maintenance.

The requirement of tryptophane for the growing rat as determined in these studies is about 0.08 per cent of the diet compared to Rose's (8) figure of 0.2 per cent. The requirement of isoleucine, on the other hand, of approximately 0.8 per cent is somewhat higher than that given by Rose which is 0.5 per cent. The above figure of 0.8 per cent compares to a figure of 0.7 per cent calculated in a previous study (1). Block (9) has calculated the amino acid requirements of the human upon the basis of the requirements of the young rat as determined by Rose (8). These studies point out the futility of such calculations.

SUMMARY

1. For the growth of rats, human fibrin is a high quality protein, while human albumin supports no growth at all when fed as the sole source of protein. Globulin is intermediate between the two.

2. For the growth of rats, human albumin is relatively deficient in tryptophane and isoleucine.

3. Maximum growth was obtained in rats with rations containing approximately 0.08 per cent *l*(-)-tryptophane and 0.8 per cent *l*(+)-isoleucine. These amounts correspond to intakes of 94 mgm. and 880 mgm. per kgm. of body weight per day, respectively.

4. In contrast to the poor nutritional quality of albumin as found in rat growth studies, nitrogen balance in the adult dog was maintained when this protein supplied only 6 per cent of the calories. It is estimated that no more than 1.6 mgm. of tryptophane and 15 mgm. of isoleucine per kgm. of body weight per day are required by the adult dog.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION. XXV. THE PREPARATION OF ANTI-A ISOAGGLUTININ REAGENTS FROM MIXED BLOOD OF GROUPS O AND B^{1,2}

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(Received for publication May 1, 1945)

In a previous publication (1), it has been stated that reagents for the determination of blood groups are obtained as products of the human plasma fractionation program. By a chemical procedure, the isoagglutinin-containing globulins are separated from most of the other plasma proteins, thereby enabling the use of the latter fractions for other purposes (2), and the use of the refined isoagglutinins in concentrated form for blood group examinations. The properties of the preparations obtained in this manner from group-specific plasma have been described (1, 3), and the United States Navy has let contracts with the industry to procure the reagents. This laboratory served to control³ this isoagglutination production program, and so it has had the opportunity of determining what changes in methods would provide larger amounts of good quality blood-grouping preparations.

The control of the products is based on comparison of their properties with the properties of Reference Standards. Two blood-grouping re-

agents, one anti-A and the other anti-B, each processed by the Harvard Pilot Plant from group-specific plasma, were adopted as Reference Standards by a committee of experts headed by Dr. E. L. DeGowin (3). Two different techniques (1) are used to compare any product with the Reference Standard of the same group. One test is designed to measure the amount of isoagglutinin by a "titration" procedure. The other technique is used to evaluate the product's "avidity," i.e., the speed of agglutination and the size of clumps obtained when the preparation is mixed with a cell suspension on a slide. Thus, preparations are judged acceptable if samples are found to be equal or superior to the Reference Standard by all tests, and if, furthermore, they are found to be "specific" for the group of cells with which they are supposed to react. Anti-B preparations are tested with B cells for titer and avidity. The existence of subgroups of the blood group A makes it necessary to test anti-A preparations both with A₁ and with A₂B cells.⁴

¹ This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

² This paper is Number 35 in the series "Studies on Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

³ The group of investigators in associated laboratories who have assisted regularly in the appraisal of these products includes Dr. W. C. Boyd, Boston University Medical School; Dr. L. K. Diamond, Children's Hospital, Boston; and Dr. E. L. DeGowin, University Hospitals, Iowa City. The laboratory of Capt. L. R. Newhouser has reported on behalf of the U. S. Navy. At the Harvard Medical School, the control group, under the direction of Dr. E. J. Cohn and Dr. J. L. Oncley, includes Mr. J. W. Cameron, Mr. P. M. Gross, Miss M. M. Haddon, Miss S. G. Miller, Dr. D. A. Richert, and Miss J. Sullivan.

THE CHOICE OF PLASMA TO BE PROCESSED INTO ISOAGGLUTININ REAGENTS

In the method previously described, the production of isoagglutinins started with the collection of group-specific plasma from random donors of a single group. It was found that anti-A preparations from pools of B plasma were often deficient in their reactions with A₂B cells. It was recognized that the isoagglutinin titers of individuals of group B, for example, vary greatly, and that the potency of a pool could be increased by omitting low-titered plasma, so an attempt was made to

⁴ The reactions of A₂B cells with anti-A preparations are, in general, much weaker than are those of A₂ cells; hence use of cells of the former group provides a more sensitive criterion for rejection of products weak in subgroup activity. We are indebted to Drs. L. K. Diamond and W. C. Boyd for calling our attention to this fact.

improve the B pools by selecting the specimens to be included. An unfortunate result of this procedure was that it decreased the amount of plasma processed into blood-grouping preparations. The yield of the reagents from a given number of blood donations is already limited by the low incidence of donors of group B (Table I). A large part of the available supply of group A plasma was not needed in this program since the routine examination for blood group necessarily utilizes both kinds of isoagglutinin in equal amounts.

TABLE I

Frequency of blood groups in the United States (4)

Blood group	Percentage of population
O	45
A	41
B	10
AB	4

We therefore investigated the plasma from group O donors. This kind of plasma is available in the largest amount, and is known to contain agglutinins for both A and B erythrocytes. Its use as a source of anti-A agglutinin^a would depend first, on the amount of anti-A antibody present, and second, on the removal of anti-B activity.

Properties of group O plasma

The results of isoagglutinin titrations of plasma samples obtained from random^a donors of groups O and B, respectively, are shown in Table II. Considering the probable factors of uncertainty of the ratios, we conclude that when enough donors are represented in the pools to minimize the effect of variations among individuals, an O plasma pool is approximately equal to a B pool in its titer against A₁ cells, and is superior to the B in its titer against A₂ cells.^{6a} These observations, which

^a We use the term "A" generically to include the subgroups A₁, A₂, etc. In a like sense, we use the term "anti-A" to include all of the antibodies effective against A cells.

^{6a} Only the following data are excluded from the computations: first, a single test representing a single O donor vs. A₁ cells, since the plasma was highly hemolytic for these cells; second, tests on plasma from a pool of bloods in which at least one A blood was included by accident.

^{6b} (Note added in proof:) A paper in the German literature (10), recently come to our attention, states that O serum usually agglutinates A₂ erythrocytes more readily than does B serum, and that O serum has more nearly the same titer vs. A₁ cells on the one hand, and A₂ cells on the other, than has B serum.

TABLE II

Comparison of O and B plasma titers

Cells used for test	Group of plasma	Number of donors represented	Number of tests performed	Results	
				Average ratio of plasma to Reference Standard ^b	Ratio of average titers, O plasma:B plasma
A ₁	O	88	31	0.24	1.3
	B	23	10	0.19	
A ₂	O	83	13	0.75	2.6
	B	22	7	0.29	
B	O	89	32	0.15	

^a Some plasmas tested were obtained from individual donors; in other cases, plasmas from as many as 12 donors were pooled.

^b Comparison with Reference Standard (104B vs. A₁ and A₂ cells; 9193A vs. B cells) is not as accurate as comparison with plasma of the other group (last column of the table), since only a few tests were done on the Reference Standards.

are supported in later results representing larger populations, indicate that less than 5 per cent of the total available plasma could be included in a pool of selected B plasma which would match the activity against A₂ cells of pooled, unselected O plasma, obtained from 45 per cent of all donors.

Removal of anti-B activity from group O plasma

There is considerable antibody in O plasma that reacts with B erythrocytes. The use of B "soluble substance" for neutralizing this antibody was considered, but it was deemed preferable to remove the undesired isoagglutinin from the system by absorption with B cells. We found it possible to remove the greater part of the anti-B activity by mixing O plasma with B cells, or by mixing O blood with B cells, or simply by mixing O blood with B blood. The absorption was found to be more rapid at room temperature than at 0° C., and also was found to be faster when larger proportions of B cells were present. The aim of the absorption, however, is not necessarily the complete removal of anti-B antibody from plasma. Later in the process, residual activity^c may be removed.

^c Part of the decrease of anti-B activity during absorption may be due to neutralization of antibody by group-specific substance present in B plasma (5) or released by B cells. Possibly fractionation of the resulting plasma could result in separation of neutralizing substance from the antibody while concentrating the latter.

e.g., by reabsorption. Since it is important that plasma resulting from the absorption procedure be suitable for processing into the other products obtained from the fractionation program, it was decided to absorb at 0° C., and to mix O and B bloods, in the same proportion as their relative incidences in the population. Experiments performed under these conditions showed that less than 5 per cent of the original anti-B activity remains after absorption lasting 10 to 15 hours, and that most of the decrease is obtained in about 2 hours.

The agglutinating properties and fractionation of absorbed plasma

The plasmas resulting from absorption experiments were found to have good anti-A activity, particularly for A₂B cells. Tested with these cells, the avidities of the absorbed plasmas were better than the A₂B avidity of the anti-A Reference Standard, and the plasmas gave no slide reaction with B cells. However, in one experiment,⁸ satisfactory plasma did not result from a mixture of blood (in this case the anti-B titer was, as usual, very low after absorption, but the anti-A₁ titer was less than 1 per cent that of Reference Standard) and here it was proved that at least one A blood, in a total number of 69, had been included by accident with the O and B bloods. Thus the importance of eliminating from the pool all cells having the A-agglutinin, *i.e.*, A and AB cells, was demonstrated.

The absorbed plasma was fractionated, and thus it was found that the solubility properties of the anti-A antibody of O plasma are similar to those of the isoagglutinins from A or B plasma. It was also shown that the remaining anti-B activity could be eliminated by reabsorption, and the resulting anti-A blood-grouping preparations were found to be specific.

LARGE-SCALE EXPERIMENTS UTILIZING O PLUS B BLOOD

It was important to show that the method could be applied to large runs, involving a number of

donors great enough to give statistical validity to the results. These experiments were carried out at the Fort Worth, Texas, Serum Albumin Plant of Armour and Co.⁹

Blood-grouping procedure

Since it had been shown that A and AB blood must be excluded from the O plus B pool, each step leading to the preparation of the pool was checked. A double blood-grouping method¹⁰ based on serology samples received from the Blood Donor Centers was adopted. The first step, the cell-grouping test, was performed on erythrocytes and utilized isoagglutinin reagents. After this preliminary sorting into blood groups was made, all the samples of each single group were retested by a serum-grouping technique, *i.e.*, samples of the individual serums were treated with specially prepared cell suspensions of known groups, and the presence or absence of agglutination was determined. In this way, errors made in the first step were found, and the corresponding samples were rejected. In some of the runs the serum-grouping procedure was also used to reject bloods whose serums hemolysed the test cells. The criteria for hemolytic rejections varied in different cases, and are shown in Table III. In the remaining runs, heated serums resulting from the syphilis-testing procedure were used for the serum-grouping test and no hemolytic rejections were made. In no case was rejection based on the strength of the agglutinating reactions obtained, so that in *this* sense no selection of specimens was made. (See next section, however.)

Blood pooling and separation of plasma

A pool of all of the group B bloods for each of the O plus B runs was prepared and then mixed with a similar pool of O bloods to start absorption. Since separation of plasma routinely was carried out at 8° C. at this plant, absorption was done at this temperature. The mixtures were stirred continuously until the end of the time allowed, and then the blood was fed into a battery of centrifuges which continuously separated plasma and cell-rich fractions. Details of the pooling and blood-separation procedure are given in Table III, which also presents the yields obtained. It will be noted that approximately 280 ml. of citrated plasma, containing about 17 grams of plasma protein, were obtained from each donor, regardless of whether O plus B or A bloods were processed. Table III also shows the number of errors detected in the serum-grouping test, and the importance

⁹ We are indebted to Dr. J. D. Porsche for making the arrangements, and to the staff of the plant, especially Mr. A. Koehler, Mr. J. H. Weare, Mr. A. F. Holtorff, Miss M. Slattery, and Mrs. A. Swan for their assistance.

¹⁰ A more detailed description of this and later parts of the procedure is included in an Interim Report submitted to the Committee on Medical Research of the Office of Scientific Research and Development.

⁸ In a run made by one of the cooperating commercial laboratories in August, 1944, the resulting "O plus B" plasma was also poor. Possibly their experience too was caused by the accidental presence of the A-agglutinin.

TABLE III
Blood grouping, pooling, and centrifugation

Blood groups	O plus B			A	
	AI-1, 188	AI-2, 190	AI-4, 192	AI-3, 190	AI-5, 192
Run number					
Bloods rejected in serum-grouping test:					
Number of errors found ^a	0	4	22	1	6
O serums causing hemolysis ^b (per cent)	40	34	0		
B serums causing hemolysis ^c (per cent)	7	4	0		
Blood-pooling: ^d					
Total number of donors represented in pools ^e	67	292	715	278	553
Ratio, O donors: B donors in pools	4.2	4.5	4.6		
Time allowed for absorption:					
Hours elapsed from mixing of O and B bloods to start of centrifugation	10	1	2		
Hours elapsed from mixing of O and B bloods to end of centrifugation	11	3.2	5.5		
Yields obtained, per donor:					
Grams red cell fraction	222	237	240	247	230
Milliliters plasma ^f	293	283	274	278	271
Grams plasma protein ^f	17.4	17.2	16.7	16.9	16.4

^a After the samples were sorted by grouping the cells, the O and B serums were tested for errors with both A and B cells. Group A serums were checked with B cells only. Bloods cell-grouped as AB were not checked; they were combined with misgrouped bloods and with bloods rejected for causing hemolysis, and were not further included in these experiments.

^b O serums which caused any hemolysis of the A cells used in serum-grouping were not included in Run AI-1, 188. For Run AI-2, 190, O serums were not used if they caused complete hemolysis of the A test cells, or any hemolysis of the B test cells. The serums for Run AI-4, 192 were tested after being heated at 56° C. for 30 minutes, and no hemolysis of test cells was observed.

^c One B serum, which hemolyzed the group A test cells, was excluded from Run AI-1, 188. For Run AI-2, 190, B serums were not used if they caused complete hemolysis of the A test cells. Heated serums were tested for Run AI-4, 192.

^d Most of the bloods were drawn less than 2 days before use, and none was older than 3 days.

^e In each of the O plus B runs, the bloods were pooled before centrifugation. Pools of blood were not made in the A runs, but the plasma was pooled in each case.

^f Volume of plasma is calculated from the weight obtained, assuming a density of 1.030 for citrated plasma. Protein concentration is calculated by subtracting 0.23 gram N per liter, assumed to be the NPN concentration, from the value obtained by Pregl analysis for the total N concentration, and multiplying the difference by 6.25. The results are expressed relative to the number of donors represented in the pools, and have been corrected for known amounts of material removed in processing.

of the double blood-grouping method is thus demonstrated.¹¹

Comparisons of the plasmas and of samples, removed at intervals from the pools and cup-centrifuged, indicated that what hemolysis was obtained could be traced to mechanical factors (*e.g.*, poor behavior of the centrifuges, or too prolonged or too vigorous stirring) and did not correlate with the presence of hemolytic plasma in the pool. Bacterial counts showed that when simple precautions were observed the plasma could be prepared free of detectable contamination.

Agglutinating properties of absorbed plasma

Isoagglutinin assays of plasma samples from the large-scale experiments are reported in Table IV. Results of titrations are presented in terms of the "titer index," which we define as the negative logarithm, to the base 2, of the highest dilution

showing agglutination.¹² Avidity measurements of the speed and extent of agglutination are reported in terms of three observations: the time required for "first visible" agglutination, that for "complete clumping," and the "size" of final clumps obtained. The appearances called "first visible" and "complete clumping" respectively are fixed in terms of the appearances observed at arbitrary times when the cells of known individuals react with the Reference Standards,¹³ and the

¹¹ One advantage of this method of expressing titration results is that the precision, about ± 1 titer index unit, is easily stated.

¹² These determinations were made by delaying the mixing of the faster preparation with its drop of cells by as much time as was needed to bring both preparations being compared to the end-point simultaneously. Repeated trials were necessary to ascertain the time delay needed, and the precision is judged to be about 5 per cent in the range 20 seconds to 200 seconds.

¹³ The evidence suggests that errors were due to mistakes of the persons doing the cell-grouping tests, rather than to weak isoagglutinin preparations.

TABLE IV
Isoagglutinin assays of plasma samples

Run	O plasma pool				B plasma pool				1-hour sample from mixed pool				Final plasma			
	T	F	C	S	T	F	C	S	T	F	C	S	T	F	C	S
Tested with A ₁ cells. Reference Standard 104B = $\bar{7}$ 5 20 lg																
AI-1	$\bar{8}$	15	55	>sm	8	12	33	md	7.5	15	55	>sm	7.5	20	80	sm
AI-2	7.5	13	45	<md	7.5	12	33	md					7	18	65	>sm
AI-4	$\bar{8}$	10	25	>md	$\bar{8}$	10	27	>md	$\bar{8}$	12	32	md	$\bar{8}$	13	45	<md
AI-3													<0	>300		
AI-5													<0	>300		
Tested with A ₂ B cells. Reference Standard 104B = $\bar{5}$ 20 75 sm																
AI-1					5	30	120	sm	$\bar{5}$	60	200	sm	5	90	>300	>mc
AI-2					4.5*	30	120	sm					5*	50	300	<sm
AI-4					$\bar{3}$	27	100	>sm	5	15	45	<md	$\bar{5}$	25	90	>sm
Tested with B cells. Reference Standard 9193A = 9 -5 25 lg																
AI-1	$\bar{7}$	20	100	md	<0	>300			1.5*	>300			0.5	>300		
AI-2	$\bar{5}$ *	50	300	sm		>300							0.5	>300		
AI-4	$\bar{6}$ *	20	90	md		>300			1.5*	>300			0.5	>300		
AI-3													6.5*	50	300	sm
AI-5													$\bar{6}$ *	50	300	sm

Symbols:

T, Titer index. An overlined number means that a weak positive reaction was obtained at the corresponding dilution. <0 means no positive reaction was obtained even with undiluted material. * means the titer index is corrected for a slightly different value obtained for the Reference Standard when the test so marked was made.

F, Seconds required for "first visible" macroscopic agglutination to appear in slide test.

C, Seconds required for "complete clumping" to appear in slide test. >300 means the corresponding degree of agglutination is not attained in 300 seconds, the maximum duration of the test.

S, Size of final clumps attained in slide test. In ascending order: mc, microscopic; sm, small; md, medium; lg, large. These symbols are modified by >, larger than, and <, smaller than.

scale of sizes is similarly arbitrarily defined experimentally.

Comparison of the assays¹⁴ on the group O plasma samples shows that the best avidity resulted when no rejection for hemolytic reaction of the serum was made, and the same conclusion is indicated for the B plasmas. Further, the avidity, tested with A₁ cells, of B plasma, where a lower proportion of hemolytic rejections was made, was better in Runs AI-1 and AI-2 than was the corresponding avidity of O plasma, from which more hemolytic specimens were omitted (compare Table III). However, in Run AI-4, where no hemolytic rejections were made from either group, it was found that O and B plasmas

were approximately equal in their avidities for A₁ erythrocytes. (This may be compared with our earlier statement regarding approximate equality of the titer against A₁ cells of pooled plasmas of the two groups.¹⁵) The avidities of B plasmas against A₂B cells and of O plasmas against B cells¹⁶ also

¹⁵ Bryce and Jakobowicz (6) report isoagglutinin determinations on 712 group O donors and 178 group B donors titrated individually against A₁ cells. After some mathematical assumptions are made, it is possible to show that their data support our conclusion that pooled O plasma is approximately equal or only slightly superior to pooled B plasma in its activity against these cells. Our data are obtained from a total of 675 group O donors and 151 group B donors and all cases where hemolytic rejections were made are excluded.

¹⁶ Comparison of tests with B cells shows that when no rejections for hemolysis of these cells by O serum were made (Runs AI-1 and AI-4), the resulting O plasma was superior to that obtained from A donors (Runs

¹⁴ These conclusions are largely based on avidity results since the titration method cannot show fine differences unless a larger number of tests are performed.

support the general conclusion that the more rejections made for hemolytic reactions with erythrocytes, the poorer the resulting pool in its avidity for these erythrocytes. Presumably hemolytic plasmas are among those with the highest individual avidities.¹⁷ Rejection of hemolytic specimens appears to be a kind of "negative selection."

The plasmas resulting from O plus B mixtures have had more than 95 per cent of the original anti-B antibody removed by the end of the absorption. However, there is in all cases a simultaneous decrease in anti-A avidity. The results, which were checked repeatedly, show that this decrease is progressive; the final plasma is poorer than the 1-hour sample. Our earlier small-scale experiments showed a similar phenomenon in respect to slight progressive decreases in titer as well as avidity.¹⁸ It has been thought (7) that in some O plasmas part of the activity against A cells may be due to a doubly reactive antibody, effective against both the A and the B agglutino-gen. Treatment of such plasma with B cells would thus result in removal of this part of the anti-A activity. However, the continued decrease of anti-A avidity between the 1-hour sample, where the anti-B activity is already very low in comparison with the anti-A, and the final plasma makes it doubtful that a doubly-reactive isoagglutinin could alone account for the entire loss obtained. A different explanation of the phenomenon has been offered by Thomsen (8), who states that erythro-

AI-3 and AI-5) in its anti-B activity. This suggests that O plasma may be a useful source of this antibody also (compare Table II). While no O plus A runs have yet been attempted, a plant processing isoagglutinins by the present method would obtain an anti-A yield some one-third larger than its anti-B output. It may be feasible to equalize production by processing O plus A runs.

¹⁷ This suggests that if hemolytic plasmas alone were chosen for processing, better material would result. The final isoagglutinin preparation would probably not be hemolytic since all of the components of complement would not be present in this fraction.

¹⁸ Not only is it extremely improbable that the A-agglutino-gen was present in every case to cause the decrease observed, but the slight drop found, contrasted with the drop to less than 1 per cent of the titer of the Reference Standard in the one experiment where the A-agglutino-gen was known to be present, makes it seem that such could hardly be the explanation. It is noteworthy that Fraction III-1-1 was later reabsorbed with B cells with no detectable loss of anti-A avidity.

cytes which have reacted with their specific iso-agglutinin may then take up other agglutinin by "secondary-binding."

In Run AI-4, where no hemolytic rejections were made, it will be noted that the 1-hour sample of O plus B plasma is superior not only to the B plasma but to the Reference Standard as well when tested with A₂B cells. In the other cases, however, due in part to rejection of hemolytic plasmas and in part to decrease in avidity during absorption, the final plasma pools were inferior to the B plasmas alone against A₂B cells, and in every case this was so when A₁ cells were used for the test; yet in every case it was possible to make satisfactory blood-grouping reagents from O plus B plasma by fractionation, although the yields in the different cases varied because of the different qualities of the plasmas.

Fractionation method

Each of the plasma pools was separately processed by Method 5 for plasma fractionation. About 26 per cent of the plasma protein nitrogen was present in the isoagglutinin-containing fraction (9), called Fraction II + III, regardless of whether O plus B or A material was processed.

The procedure used for the subfractionation of II + III, Method 7, wasted the prothrombin and the protective antibodies, and will, therefore, be supplanted, so it will here be described only briefly. The isoagglutinins were precipitated¹⁹ under these conditions: pH 6.35, ionic strength 0.005, room temperature, volume 10 times that of the original plasma,²⁰ and ethanol concentration below 0.1 per cent. The precipitate so obtained, called Fraction III-1-1, settled out and was dried from the frozen state. The powder was then redissolved in neutral, isotonic buffer, in a volume one-fifth that of the original plasma, and reabsorbed with group B red cells. Five ml. of the cells, packed by bucket-centrifugation, were used for each liter of plasma represented. The mixture of cells and redissolved Fraction III-1-1 was stirred at 0° C. continuously for 12 ± 4 hours, after which the supernatant, obtained by bucket-centrifugation, was treated with an equal volume of 0° C. water and filtered through prepared filter-pads. It had been noted earlier that losses of activity could result from exhaustive filtration, but assays showed

¹⁹ For these experiments, the method, devised by Dr. J. L. Ondley and Mr. J. W. Cameron, was modified with the assistance of Dr. D. A. Richert.

²⁰ Only portions of the Fractions II + III from each of the plasmas AI-2, 3, 4, and 5 were subfractionated, and calculations were made accordingly. In these cases, the numeral 1 is suffixed to the plasma numbers to indicate the products of subfractionation.

TABLE V
Isoagglutinin assays of solutions^a of the intermediate products of fractionation

Run	Fraction III-1-1 (5-fold over plasma) ^a				Reabsorbed III-1-1 (5-fold over plasma)				Fraction ^b III-1-2 (20-fold over plasma)			
	T	F	C	S	T	F	C	S	T	F	C	S
Tested with A ₁ cells. Reference Standard 104B = $\bar{9}$ 5 20 lg												
AI-1					$\bar{9}$				10.5	6	27	lg
AI-21	$\bar{9}$	12	32	md	8	12	32	md	10	6	22	>lg
AI-41	10	5	18	lg	10	5	18	lg	10	<5	15	>lg
AI-31									<0	sp		
AI-51									<0	sp		
Tested with A ₂ B cells. Reference Standard 104B = $\bar{5}$ 20 75 sm												
AI-1					$\bar{6}^*$				$\bar{7}$	15	60	<md
AI-21	5*	50	300	<sm	$\bar{5}^*$	50	300	<sm	7*	15	55	<md
AI-41	$\bar{7}$	15	50	<md	$\bar{7}$	15	50	<md	8.5	7	25	lg
Tested with B cells. Reference Standard 9193A = 9 5 25 lg												
AI-1					0				0	sp		
AI-21	<0	>300			<0	>300			0	sp		
AI-41	2	>300			<0	>300			<0	sp		
AI-31	$\bar{7}$	15	60	md					9	<5	18	>lg
AI-51	8	15	60	md					9	<5	18	>lg

Symbols:

sp: Slide tests for specificity showed no agglutination of the cells indicated, or of group O cells, in 7 hours. The slides were stored in a moist chamber at room temperature and were observed microscopically at intervals.

For other symbols, see Table IV.

^a All solutions tested were of neutral pH, and isotonic. Their concentration is expressed as "*n*-fold over plasma," where *n* is equal to the volume of plasma from which the aliquot is obtained divided by the volume of the assayed solution of this aliquot. The yield of isoagglutinin in any fraction may be calculated if this quantity and the titer indexes (T's) are known.

^b Aliquots of the wet III-1-2 precipitates were tested at 1/20 plasma volume in order to determine what concentration would be needed in each case for the final solution (see Table VI) to exceed the corresponding Reference Standard.

that in these experiments the filtration procedure did not cause detectable loss of activity. The filtrate was reprecipitated under the same conditions as described for the precipitation of Fraction III-1-1. The small amount of hemoglobin introduced during the reabsorption was thus removed, and the resulting Fraction III-1-2 was white. About 3 per cent of the plasma protein nitrogen was obtained in this fraction, *i.e.*, about one-half gram of protein ($N \times 6.25$) per donor.

Isoagglutinin assays of the products, and the yields obtained

While the yields of isoagglutinin obtained by this fractionation procedure were not quantitative, it will be noted that the antibody from O plus B plasma fractionated in the same way as did that from group-specific A plasma, that reabsorption was carried out without loss, and that satisfactory final material resulted. These conclusions are

justified by the results reported in Tables V and VI. It will be noted that each of the preparations was specific for the group of cells with which it was supposed to react.

The yields of the blood-grouping reagents obtained in the different O plus B runs increased in the order of the qualities of the plasmas. Table VI shows that, in runs where no exclusion for hemolytic reactions of serum was made, each blood donation processed furnished enough of the corresponding reagent for more than 250 tests. Since routine blood group determinations require equal quantities of the anti-A and the anti-B reagents, the amount of material that may be produced from a given number of blood donations is limited by the incidence of A donors, unless it proves feasible to increase the yield even further by the processing

TABLE VI

Yields and properties of the blood-grouping reagents

Product number	Amount obtained from each donor ^a	Tests on final solution ^b			
		T	F	C	S
Tested with A ₁ cells.					
Reference Standard 104B =		9̄	5	20	lg
AI-1 (anti-A)	7.4 ml.	11.5	<5	17	>lg
AI-21 (anti-A)	10.6	10	<5	17	>lg
AI-41 (anti-A)	14.0	10	<5	14	>lg
Tested with A ₂ B cells.					
Reference Standard 104B =		5̄	20	75	sm
AI-1 (anti-A)		8̄	10	35	<md
AI-21 (anti-A)		7	12	45	<md
AI-41 (anti-A)		8.5	7	25	lg
Tested with B cells.					
Reference Standard 9193A =		9	5	25	lg
AI-31 (anti-B)	13.9 ml.	9	<5	18	>lg
AI-51 (anti-B)	13.6	9	<5	18	>lg

Symbols:

See Table IV.

^a The finished product is a dried powder. It is prepared by desiccation from the frozen state of a solution containing an isotonic mixture of sodium phosphates and chloride, neutral in pH, and containing Fraction III-1-2 protein in such a concentration as to provide the required isoagglutinin activity. The product may be dispensed in whatever quantities are convenient; for example it may be packaged in such amounts as to provide vials containing 1 ml. of blood grouping reagent when reconstituted with distilled water (or with the diluent containing dye and preservative specified in Navy contracts). The figure listed in the table is the number of 1 ml. vials obtained from each blood donation processed. A pair of such vials, one of each group, if used economically, is sufficient for about 20 routine blood group determinations.

^b Titer indexes given here are calculated from the results obtained by titration of the aliquots tested at one-twentieth of plasma volume (Table V). The avidity measurements were made on the final solution dried.

of O plus A runs (see footnote 16). If O plus A runs are not processed, a plant receiving 1000 blood donations could produce isoagglutinins from about 800 of these bleedings, and could thus manufacture by the present method about 5000 one ml. vials of each group, a quantity of each reagent sufficient for about 100,000 blood-grouping determinations, provided that the reagents are used economically in the examinations. A number of other products (2) may simultaneously be obtained from these blood donations.

Perhaps the most notable property of the material processed from O plus B blood is that it shows

relatively little disparity between its reactions with cells having the A₁- and the A₂-agglutinogens, respectively. By contrast, there is a wide difference in reactivity against these different cells in preparations made from group-specific B plasma,²¹ exemplified by the Reference Standard itself (see Tables V and VI). In routine blood group examinations, therefore, all subjects having the A-agglutinin, regardless of their subgroup, will react in more nearly the same time if the O plus B reagent is employed, and, presumably, the accuracy of such determinations may thereby be increased.

Expert appraisal of the blood-grouping reagents

Samples of each of the final products were submitted to a number of investigators experienced in the evaluation of such material. Their appraisals are indicated in Table VII. Each investigator employed his own procedures, and his results are therefore compared with his simultaneous determinations of the activities of the corresponding Reference Standards.

SUMMARY

Pooled plasma from group O donors, available in larger amount than that from group B donors, is superior to the latter in its activity for red cells having the A₂-agglutinin, and is about equal in its activity for A₁ red cells. The anti-B activity of group O plasma may be removed. Specific anti-A globulin preparations with satisfactory agglutinating properties have been produced from mixtures of O plus B blood by methods which are applicable to the large-scale commercial production of blood-grouping reagents.

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²¹ In this respect, the O plus B material approaches isoimmune B plasma (prepared by injection of human subjects with soluble group substance), which Dr. W. C. Boyd has shown to react about as well with the A₁ as with the A₂-agglutinogen.

TABLE VII

Expert appraisal of the blood-grouping reagents, compared with Reference Standards^a

Investigator ^b	Prep. numbers	vs. A ₁ cells				vs. A ₂ B cells ^c				Specificity	Comment
		Rel. titer	Rel. avidity			Rel. titer	Rel. avidity				
			(F)	(C)	(S)		(F)	(C)	(S)		
WCB	AI-1	0.5	2.7	2.5	=	0.5	2.5	2.5	>	No reaction with B or O cells	These preparations, especially AI-41, entirely satisfactory
	AI-21	1	2.7	2.5	<	0.5	2	2.1	=		
	AI-41	1	2.7	2.8	=	8	2.5	2.5	>		
JWC & DAR	AI-1	2	1.3	1.3	>	4	2.5	2.1	>		
	AI-21	1	1	1	>	3	1.5	1.2	>		
	AI-41	2	1	1	=	6	2.5	2.4	>		
LKD	AI-1	4	1.4	1.3	=	16	2.4	2.6	>	No reaction with B or O cells	Excellent Good Best of group
	AI-21	4	1.2	0.7	<	8	1.7	2	=		
	AI-41	8	1.8	1.4	=	64	5.5	4.5	>		
		vs. B cells									
WCB	AI-31	0.5	0.8	1.1	=					No reaction with A or O cells	Quite satisfactory
	AI-51	0.1	0.8	1.1	<						
JWC & DAR	AI-31	1	1.2	1.1	>						
	AI-51	1	1.2	1.1	>						
LKD	AI-31	8	1.5	1.6	=					No reaction with A or O cells	Excellent testing serum Good
	AI-51	4	1.3	1.3	<						

^a The "relative titer" is the ratio between the titer found for the preparation and that found in simultaneous test of the Reference Standard. "Relative avidity" included (F), the ratio between the time required for the Reference Standard to give "first visible" agglutination and the time for the preparation to reach this stage; (C), similarly defined for "complete clumping"; and (S), where we denote whether the preparation gives equal (=), larger (>), or smaller (<) final clumps compared with those given by the Reference Standard. The anti-A preparations were compared with Reference Standard 104B; the anti-B preparations with 9193A.

^b Investigators: WCB, Dr. William C. Boyd; JWC & DAR, Mr. James W. Cameron and Dr. Dan A. Richert; LKD, Dr. Louis K. Diamond.

^c The preparations tested were samples of the dried product reconstituted before use.

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CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION. XXVI. THE PROPERTIES OF SOLUTIONS OF HUMAN SERUM ALBUMIN OF LOW SALT CONTENT^{1, 2}

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Whole plasma is distributed as a dry powder in the interests of preserving its labile constituents. The albumin, the γ -globulin, the fibrinogen, the thrombin, and most of the other products of plasma fractionation are prepared as dry powders and could also be distributed in the solid state. Separated from each other, purified of the enzymes of plasma, many of them may be distributed, however, as stable concentrated solutions whose specific natural properties, both chemical and biological, remain unaltered for protracted periods of time.

In the fractionation process (1), each of the plasma proteins is precipitated in the neighborhood of its isoelectric point. The dry proteins can be dissolved at any concentration or in any diluent. Since the plasma proteins differ widely in their chemical properties and in their natural functions, the best diluent for each depends both upon these properties and upon the clinical uses for which the solution is prepared.

Solutions intended for clinical use should generally have a pH not too far from that of the blood; a viscosity not much greater than that of blood; an essentially isotonic concentration of solute molecules, which should, moreover, be chosen so as to enhance to any necessary level the stability of the dissolved proteins. Neutral isotonic sodium chloride has been found less effective in increasing the thermal stability of purified plasma proteins than certain other diluents. Thus

the stability of the γ -globulins is not greatly influenced by sodium chloride but is increased by sugars, by glycine, and by certain other non-electrolytes. Glycine has been adopted as the diluent in which γ -globulin is now distributed to the Armed Forces and to the American Red Cross because it gives a sufficient stability, because it is not toxic, and because it is not a foreign substance, but is a native constituent of the body. Although γ -globulin solutions are more stable in higher concentrations of glycine,³ a 0.3 molal solution has been chosen, since it is isotonic and, therefore, more satisfactory for injection.

Human serum albumin was developed as a blood derivative for the treatment of shock. The molecules of this plasma protein are large enough to be held in the blood stream but small enough to have a high osmotic efficiency,⁴ and are so symmetrical that a 25 per cent solution is isoviscous with blood and so stable that it may be dispensed in aqueous solution:

The first normal human serum albumin pro-

³ It may prove unnecessary to stabilize γ -globulin sufficiently to permit a high temperature treatment for the destruction of viruses since antibodies against the virus of infectious hepatitis have been shown to be present in γ -globulins from large pools of plasma in sufficient quantities to give protection against the disease (2). Moreover, such a treatment could not be recommended without proof that bacteria and viruses are less stable to heat than the antibodies.

⁴ Osmotic pressure is used in its classical sense to designate the equilibrium pressure across a membrane with about the same permeability as the capillary walls (3). This is sometimes called the oncotic, or colloid osmotic, pressure. The osmotic efficiency of a material is proportional to the volume of solution at the osmotic pressure of plasma which is produced by unit weight of that material. Osmotically equivalent quantities of two materials are those quantities which produce the same volume of solution at the osmotic pressure of plasma.

¹ This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

² This is paper No. 36 in the series, "Studies on Plasma Proteins," from Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

duced by the Harvard Plasma Fractionation Laboratory was a 25 per cent solution of nearly isoelectric albumin in water (4). This concentration was chosen to give the smallest possible volume for transport without increasing the viscosity so much as to render inconvenient the passage through bacteriological filters or injection through an intravenous needle. Very soon, however, the pH was adjusted to approximate neutrality in order to meet the objections to the infusion of an acid solution, and 0.15 mole sodium chloride per liter was added to render the solution isotonic.⁶ As in the case of plasma, merthiolate or a similar mercurial bacteriostatic agent was introduced at the request of the National Institute of Health when distribution for the Armed Forces began. Later, the sodium chloride content was increased to 0.3 mole per liter (6) in order to render a product distributed in solution more stable at high temperature such as might be expected to be encountered under some military conditions.

The use of normal human serum albumin as a diuretic agent (7) has led to a need for a preparation which contains no mercury and but little sodium ion. Solutions of isoelectric albumin without other added solute have been prepared for experimental studies (8 to 10). Such solutions are strongly hypotonic and hemolyze red cells if injected rapidly without the addition of glucose or other solute to reduce hypotonicity. Such solutions are also acid and unstable. If it were important to inject sodium-free serum albumin, it could be prepared and distributed, as is plasma, as a sterile dry powder with sufficient glucose or other appropriate solute to render the reconstituted solution isotonic, or the isotonic glucose could be distributed in the diluent container. In either case, the package would occupy more than twice the volume of the present container, the solution would still be acid, and the advantage of distributing a stable solution would be lost.

Experimental studies have thus far failed to demonstrate any great advantage in an albumin

solution from which the last traces of sodium have been removed. Indeed, the amount of sodium required to neutralize albumin, and thus to avoid injection of an acid solution, is small in comparison to the total sodium intake of a patient on a salt-poor diet (8). Such albumin solutions, neutralized with sodium bicarbonate, and in certain studies rendered isotonic with glucose, have also been prepared. They are far more stable than the isoelectric albumin solutions but less stable than the standard albumin solutions first developed for the Navy. Such salt-poor neutral sodium⁶ albuminate solutions are also far less hypotonic than the isoelectric albumin though far lower in sodium ion than the present standard albumin solution and lower still than the osmotically equivalent volume of plasma.

One hundred ml. of 25 per cent normal human serum albumin is osmotically equivalent to 450 ml. of plasma. This quantity of plasma contains about 1.6 grams of sodium ion.⁷ The present standard albumin solution, which is 0.3 molal in sodium chloride, contains far less sodium ion, 0.9 gram per 100 ml., and of this amount only 0.1 gram is required to neutralize the albumin. A further 0.1 gram is required to neutralize the other acids, mostly acetic, which are carried down with the isoelectric albumin precipitate. The latter quantity could be largely eliminated by a further purification of albumin carried out during processing. However, this quantity is small in comparison with the 0.7 gram of sodium ions added to increase the thermal stability of albumin solutions to the present high standard. This amount of sodium ion can be greatly reduced by replacing sodium chloride with other reagents which would satisfy the conditions of thermal stability and tonicity at much lower sodium concentrations.

THERMAL STABILITY OF ALBUMIN SOLUTIONS

The profound influence of salts of non-polar anions in increasing the thermal stability of serum

⁶ The tonicity of a solution is most conveniently measured as the ratio of its freezing point depression to that of plasma, 0.56° C. (5). Thus a solution with freezing point depression less than 0.56° C. is hypotonic; one with a greater depression is hypertonic. The tonicity depends upon all the solute molecules, and not upon the colloids alone.

⁷ It is possible that a stable, neutral, sodium-free solution might be obtained by substituting some other cation for the sodium ion, but no more desirable cation has yet been suggested.

⁸ The osmotically equivalent quantity of the present citrated plasma contains an additional 0.5 gram of sodium ion from the added sodium citrate, making the total sodium about 2.1 grams.

albumin has been beautifully demonstrated by Luck and his co-workers (11, 12). In the aliphatic series, this influence increases with the length of the paraffin chain, being greater for butyrate than for acetate and far greater for caprylate. Other non-polar anions, such as phenylacetate and mandelate, have been shown to be at least as satisfactory as the lower members of the aliphatic series. We have also found that sodium hippurate and other non-polar ions have a similar stabilizing action.

The replacement of 0.3 molal sodium chloride with 0.04 or 0.05 molal sodium phenylacetate or mandelate increases from 4 to 10 times the length of time during which serum albumin can be subjected to high temperatures. Moreover, this substitution reduces to one-sixth or one-seventh the amount of sodium employed in increasing the thermal stability of the protein.

The structural similarity of phenylacetic and mandelic acids to acetylphenylalanine, a simple derivative of a natural amino acid, was pointed out,⁸ and it was suggested that the sodium salt of this acid might also have an influence on the thermal stability of serum albumin. This suggestion has been tested by experiment and confirmed. Substitution for acetylphenylalanine of the acetyl derivative of an amino acid in which albumin is deficient for nutrition was suggested by one of us (13). Crystallized human serum albumin contains but 0.19 per cent tryptophane (14) and must be supplemented by both tryptophane and isoleucine to give satisfactory growth when fed to young rats (15 to 17). Whole plasma also is deficient in isoleucine though not in tryptophane (18).

There are thus a large number of non-polar anions that are effective stabilizing agents for serum albumin solutions. Butyric acid and the next members of this series have strong odors. Phenylacetate and caprylate are more toxic than the others⁹ though none of these substances appears to be toxic except when injected in large

amounts. Sodium mandelate, acetylphenylalaninate and acetyltryptophanate are reported not to differ markedly from each other from the point of view of toxicity or of stability. Sodium mandelate and d-acetyltryptophanate are presumably excreted, whereas l-acetyl tryptophanate appears on the basis of the evidence thus far available (15, 19) to be utilized by the animal body. These are the non-polar anions which have thus far been used in the most extensive experiments in man (8).

Various methods have been used to follow the influence of reagents on the thermal stability of albumin solutions.¹⁰ The ultracentrifuge is used to determine the absence of any appreciable amount of aggregation into larger protein complexes or decomposition into elements of smaller molecular weight (20). Aggregation and decomposition, if they involve a large proportion of the dissolved molecules, are also reflected by changes in osmotic pressure (3). Incipient denaturation in these solutions, however, has generally been accompanied by an aggregation of too small a number of molecules to be noted by these methods. It is, however, readily observed by changes of viscosity or by the appearance of haze in the solutions, and can be most conveniently measured in a nephelometer (6). The clarity of albumin solutions after passage through appropriate bacteriological filters has generally been about 10 nephelometric units.¹¹ An increase of 20 nephelometric units brings these solutions to the range in which haze is barely perceptible to the eye. The haze is much less than that in reconstituted plasma, and there is no evidence that it is in any way harmful in either case, especially since there is a filter in each transfusion line to remove any large particles.

judgment regarding the usefulness of caprylic acid will be postponed until their completion. These results will be reported elsewhere.

¹⁰ In the case of γ -globulins, the limiting factor is of course not the stability of the molecule, but of the configuration upon which antibody titer depends. In the case of prothrombin and thrombin, and of the various hormones and enzymes of plasma, the stability of the native principle must be considered, as well as that of the protein molecule.

¹¹ The unit adopted in these measurements is approximately 1 per cent of the turbidity of a standard 1 per cent absolute turbidity is given as 0.0193.

* We are indebted to Dr. Hans T. Clarke for this suggestion, made at a meeting of the Protein Committee of the National Research Council.

⁹ Studies on toxicity of these reagents that have been carried out by Dr. J. Murray Luck and his co-workers and also by Dr. Otto Krayer are continuing, and final

However, haze may be employed as a quantitative measure of incipient protein denaturation.

The further aggregation of protein leads in time to the appearance of a gel in the solution. The relation between the number of days required for an increase of 20 nephelometric units as a result of heating at 57° C. to that required for incipient gelation at this temperature is graphically represented for 150 preparations of normal human serum albumin in a previous communication (6). On the average it took about 3 times as long to observe incipient gelation as to observe nephelometrically the first visible haze.

The measurements that have now been carried out in order to determine the influence of various stabilizing agents are presented in Tables I, II and III. The methods were the same as those used previously (6) but with standard human serum albumin (1) instead of crystallized albumin. The stabilizing agent was added as the sodium salt to prevent denaturation of the albumin. The albumin was neutralized with NaHCO₃ except in the solutions described in Table II and in the last column of Table III, Albumin B. All solutions were filtered through asbestos sterilizing filter pads (Seitz, serum No. 3). Studies were

carried out at 57° C., 60° C., and 63° C., and the times necessary for the increase of 20 nephelometric units and for gelation are both recorded. Gelation seems to be less affected than turbidity by the very active stabilizing agents, and the ratio of times decreases from three to almost unity for the extremely stable solutions.

In dilute solutions of the diluents, it is shown in Table I that the stabilizing action increases more rapidly than the reagent concentration. In higher concentrations, however, the stabilizing effect approaches a limiting value. The effects of sodium acetyltryptophanate and of mandelate are nearly the same, the stability in 0.04 molal solution being 2 or 3 times that in 0.3 molal sodium chloride. Sodium caprylate has a somewhat larger effect in 0.01 molal and a much larger effect at higher concentrations.

By either method of estimating incipient denaturation, the pH of optimum stability at these temperatures and in the diluents now reported appears from Table II to be at reactions close to neutrality. In the presence of these diluents and at these temperatures, the pH range previously specified, 6.8 ± 0.2 , still appears to be the optimum.

TABLE I
Effect of stabilizer concentration on stability

Reagent	pH	Method of measuring denaturation	Moles of reagent per liter					
			0.01	0.02	0.03	0.04	0.08	0.3
			time in hours at 57° C.					
Albumin A								
Sodium chloride	6.8	Nephelometry ^a						81
Sodium acetyltryptophane	6.65	Nephelometry ^a	24	68	120	225		
Sodium mandelate	6.8	Nephelometry ^a	25	71	120	215		
Sodium caprylate	6.65	Nephelometry ^a	40	480				
Albumin C								
Sodium chloride	6.8	Nephelometry ^a						38
Sodium acetyltryptophane	6.8	Gelation	70	120		390	520	
		Nephelometry ^a	19	40		180	430	
Sodium mandelate	6.8	Gelation	34	120		290	670	
		Nephelometry ^a	11	35		129	450	
Sodium caprylate	6.8	Gelation	220	820		1400	2400	
		Nephelometry ^a	70	470		1300	2100	

^a Time in hours to increase 20 nephelometric units.

TABLE II

Effect of hydrogen ion concentration on stability of human serum albumin

A. Solutions containing 0.04 mole sodium acetyltryptophanate and 0.1 mole isoleucine

Temperature	Method of measuring denaturation	pH				
		6.6	6.8	7.0	7.2	7.5
		time in hours				
57° C.	Gelation Nephelometry ^a	350 106	350 122	350 146	320 164	290 142
60° C.	Gelation Nephelometry ^a	80 24	80 27	80 32	70 30	50 22
63° C.	Gelation Nephelometry ^a	12 3.2	12 3.4	12 3.4	10 3.3	7.5 2.2
		time in hours after 10 hours at 60° C.				
57° C.	Gelation Nephelometry ^a Nephelometry ^b	300 94 72	300 84 70	300 123 96	280 110 96	76 64

B. Solutions containing 0.04 mole sodium acetyltryptophanate

		pH			
		6.3	6.9	7.3	7.6
		time in hours after 10 hours at 60° C.			
57° C.	Gelation Nephelometry ^a Nephelometry ^b	340 27 18	350 107 89	320 120 110	220 57 48

^a Hours to increase turbidity by 20 nephelometric units.

^b Hours to increase turbidity to value 20 nephelometric units above that before heating at 60° C.

These results may also be employed in calculating the average temperature coefficient of stability. The length of time that albumin solutions maintain the same stability at these temperatures is approximately halved by each increase of 1°C. The average temperature coefficients vary from 1.6 to 1.9 for the range 57° C. to 60° C. and from 2.0 to 2.2 for the range from 60° C. to 63° C. The temperature coefficient appears to increase slightly with increase in temperature and also with increase in alkalinity.

HEAT TREATMENT OF HUMAN SERUM ALBUMIN IN FINAL CONTAINERS

For a long time there has been a search for some substitute for mercurial bacteriostatic agents

in plasma and in albumin solutions. Not only do they appear to be much less effective in concentrated protein solutions than in many other media, but also a limiting factor in the amount of albumin, as of plasma, that can be safely injected in certain conditions may become the toxicity of the mercurial added as a preservative. A heat treatment similar to pasteurization seems to offer a simple way of eliminating to a considerable degree the necessity of a mercurial. It has the further advantage of destroying viruses, which might be present, as well as bacteria.¹²

There is very little information upon which to determine the heat treatment necessary to destroy bacteria or viruses. Preliminary experiments¹³ indicate that vaccinia virus is completely destroyed in 1 hour at 57° C. or 3.5 minutes at 68° C. in the presence of sodium mandelate and probably in the presence of sodium phenylacetate. Some results (21, 22) indicate, however, that the activity of tobacco mosaic virus is destroyed much more slowly. The rate of destruction is approximately doubled for each degree rise in temperature, just as for the denaturation of human serum albumin. We have studied the effect on the 57° stability of heating 10 hours at 60° C. albumin solutions with 0.04 molal sodium acetyltryptophanate or 0.04 molal sodium mandelate as stabilizing agents, and the results are shown in Tables II and III. As measured by either method, the stabilities are still greater than that of the same albumin with 0.3 molal sodium chloride as diluent. The albumin sample which we have studied most thoroughly may be heated 17 hours at 60° C. with 0.04 molal sodium acetyltryptophanate before its stability is reduced to that with 0.3 molal sodium chloride. It may be found that for the destruction of some viruses or bacteria, it is desirable to heat for a shorter time at a higher

¹² An alternative method for the destruction of viruses and bacteria might be the addition of formalin to the final albumin solution, which also increases the thermal stability. Experiments with 25 per cent solutions of crystallized bovine albumin have shown that concentrations up to 0.6 per cent do not change the immunological properties, as judged by serological tests and the sensitization of guinea pigs, or greatly alter the physical chemical properties.

¹³ We are indebted to Dr. John E. Hunter for the personal communication of these results.

SCATCHARD, STRONG, HUGHES, ASHWORTH, AND SPARROW

TABLE III

Effect of various salts on stability of normal human serum albumin

STRONG, HUGHES, ASHWORTH, AND SPARROW
TABLE III
Effect of various salts on stability of normal human serum albumin

Albumin B		Diluent						
Temperature 0° C.	Method of measuring denaturation	0.3 mole sodium chloride pH 6.6	0.01 mole so- dium acetyl- tryptophanate pH 6.8	0.01 mole so- dium acetyl- tryptophanate + 0.1 mole isoleucine pH 7.05	0.01 mole sodium mandelate pH 6.9	0.01 mole sodium mandelate + 0.1 mole glycine pH 6.85	0.01 mole sodium mandelate + 0.1 mole glycine (no carbonate) pH 6.8	
57° C.	Gelation Nephelometry ^a	time in hours						
60° C.	Gelation	47	420 184	380 176	380 130	310 109	340 123	
63° C.	Nephelometry ^a Nephelometry ^a	3.1	120		100	100	100	
			36 3.7	31 2.5	26 3.0	25 3.5	24 3.2	
57° C.	Gelation Nephelometry ^a Nephelometry ^b	time in hours after 10 hours at 60° C.						
		380 152 135	340 136 127	310 88 76	290 87 73	310 95 84		
Albumin C		Diluent (pH = 6.8)						
		0.3 mole sodium chloride	0.04 mole sodium acetyltryp- tophanate	0.04 mole sodium mandelate	0.02 mole sodium caprylate	0.04 mole sodium caprylate	0.03 mole sodium acetyltryp- tophanate + 0.01 mole sodium caprylate	0.02 mole sodium acetyltryp- tophanate + 0.02 mole sodium caprylate
57° C.	Gelation Nephelometry ^a	time in hours						
60° C.	Gelation Nephelometry ^a	120 37	390 180	290 129	820 470	1400 1300	650 420	890 720
63° C.	Gelation Nephelometry ^a	2.2	100 22	100 18	210 75		190 74	256
			13 2.5	13 2.5	12		10	45
57° C.	Gelation Nephelometry ^a Nephelometry ^b	time in hours after 10 hours at 60° C.						
		100 80	220 63 45	700 363 335	1400 1300 1300	650 370 350	890 720 710	

^a Hours to increase turbidity by 20 nephelometric units.
^b Hours to increase turbidity to value 20 nephelometric units above that before heating at 60° C.

temperature. Ten hours at 60° C. are equivalent to 5.5 hours at 61° C., 2.8 hours at 62° C., or 1.3 hours at 63° C. For the destruction of organisms, it may be desirable to heat at a higher temperature.

If it should be

^a Hours to increase turbidity by 20 nephelometric units.

^b Hours to increase turbidity to value 20 nephelometric units above that before heating at 60° C.

temperature. Ten hours at 60° C. are equivalent to 5.5 hours at 61° C., 2.8 hours at 62° C., or 1.3 hours at 63° C. For the destruction of sporulating organisms, it may be desirable to divide the high temperature treatment into 2 parts separated by a period of incubation at 37° C. The effect upon the albumin will depend upon the total time of heating.

If it should become desirable to give a more vigorous heat treatment in order to destroy some viruses or bacteria, it will be possible to make the albumin more stable at the cost of a somewhat higher sodium content by increasing the concentration of stabilizing agent. The limiting factor will presumably be the toxicity, and it will probably be more efficient to use a mixture of reagents.

which will be eliminated by the body in different ways. It is probable that a little caprylate added to the sodium acetyltryptophanate will be very useful if extreme stability becomes important.

From Table III, it is evident that a mixture of 0.02 molal acetyltryptophanate and 0.02 molal caprylate gives a greater stability at 63° C. than does 0.04 molal acetyltryptophanate at 60° C. The sodium ion concentration is the same, and the tryptophane is sufficient to make the albumin complete for growth in rats (15 to 17). It should be practicable to heat this solution 10 hours at 64° C.

TONICITY OF ALBUMIN SOLUTIONS

The 25 per cent albumin solution containing 0.3 molal sodium chloride is very hypertonic. Its freezing point depression is about 3 times that of plasma although the ratio of total solutes to osmotically active solutes is only half that of plasma because of its very high concentration. The low salt albumins, on the other hand, are distinctly hypotonic. That containing 0.3 gram per cent sodium has a freezing point depression three-quarters that of plasma, and removal of the acetate may be expected to reduce the depression to half that of plasma. The isoelectric albumin with water as diluent is extremely hypotonic. Table IV shows the sodium content, and the freezing

point depression of several albumin preparations, as well as their effect on human red blood cells. Only the isoelectric albumin solutions laked the cells.

The addition of d,l-isoleucine at a concentration of 0.1 molal to 25 per cent albumin containing 0.04 molal acetyltryptophanate is reported to make the solution complete for growth in young rats (15 to 17), and will increase the freezing point depression to 0.9 to 1.2 times that of plasma without change in the sodium content or in the stability of the albumin. The use of such a diluent has the further advantage that the alkali necessary to neutralize the albumin and the absorbed acids can be added as a buffer mixture of sodium isoleucinate and isoleucine together with the sodium acetyltryptophanate. This makes unnecessary the use of sodium bicarbonate to prevent excessive local alkalinity during the neutralization and eliminates the possibility of a shift in pH due to loss of carbon dioxide. The stabilities of such solutions are presented in Table II. These advantages, other than the possible nutritional value, can also be obtained from glycine.

THE NEW STANDARD ALBUMIN SOLUTION

There is a great advantage in having an albumin solution which either can be infused alone or can be used combined with any appropriate diluent

TABLE IV
Sodium content and tonicity of albumin solutions

Albumin type	Diluent	Number	pH	Sodium <i>grams per cent^a</i>	Freezing point ^c de- pression ^b	Percentage change in volume of packed red cells ^a		
						I	II	III
Standard	0.3 mole NaCl	93R	6.8	0.97	1.76	-17	-24	-32
Standard	0.3 mole NaCl	97R ₂					-18	-22
Low salt	0.04 mole sodium acetyl tryptophanate	153	7.3	0.32	0.47	+19	+21	+11
Low salt	0.04 mole sodium acetyl tryptophanate	158	7.0	0.29	0.42	+29	+29	+18
Low salt	0.04 mole sodium acetyl tryptophanate	158H ^d	7.0	0.29	0.42	+27	+26	+16
Salt-free	none	149	4.8	0.00	0.08	laked	laked	laked

^a Measurements made by Miss K. Fahey in the laboratory of Dr. James L. Gamble, Children's Hospital, Boston.

^b Measurements made by Dr. Alexander Brown and Miss J. Weeks. The freezing point depression of plasma is 0.56° C. (5).

^c Measurements made by Dr. Frank L. Plachte in the laboratory of Dr. Charles A. Janeway, Children's Hospital, Boston: 2 ml. of heparinized blood mixed with 2 ml. of 25 per cent albumin solution; incubated at 37° C. for 1 hour; centrifuged for 2 hours at 2500 RPM; supernatant plasma examined for hemolysis. Control tubes contained 2 ml. of heparinized blood mixed with 2 ml. of the donor's plasma. Determinations were made with three different levels, I, II and III.

^d 158 heated ten hours at 60° C.

for a particular therapeutic use. Such combination was foreseen at an early date, and the standard Army-Navy double-ended bottle was designed¹⁴ to permit the intravenous administration of saline, glucose, or other solution with the albumin. It has sometimes been found even more convenient in the field to mix 2 fluids by inserting the needle attached to the container of 1 solution into the rubber tube through which the other is being injected.¹⁵ Sufficient saline or glucose may readily be added so that the average albumin concentration is isosmotic with plasma or even more dilute. This may be desirable in severe shock or in marked dehydration. If it is desired to administer a large amount of base, it can be added as an isotonic solution of sodium lactate or other similar salt. Although it is possible to increase the ratio of sodium to albumin to any extent by simultaneous injection, it is not possible to decrease it in this way below the ratio in the original albumin solution, and a low ratio appears desirable if the albumin is to be used as a diuretic. A solution of 25 per cent albumin at pH 6.8 with 0.04 molal sodium acetyltryptophanate and 0.1 molal isoleucine if indicated,¹⁶ without a mercurial bacteriostatic agent, but pasteurized 10 hours at 60° C. or an equivalent time at some other temperature, has therefore been recommended, not only as a diuretic agent, but also to replace for use in shock the sodium chloride containing albumin heretofore available. On the basis of the accumulated chemical and clinical evidence, the Navy is now procuring albumin according to these specifications.

SUMMARY

(1) Normal human serum albumin, like other products of plasma fractionation, is separated near its isoelectric point and prepared as a dry white powder. It can, therefore, be redissolved at any concentration and distributed in any diluent.

¹⁴ The package adopted by the Armed Forces for the standard 25 per cent albumin solution was developed by Captain Lloyd R. Newhouser, Medical Corps, United States Navy and Lt. Col. Douglas Kendrick, Medical Corps, United States Army.

¹⁵ This method has been of greatest utility in the supplementing of whole blood infusions with concentrated albumin solutions.

¹⁶ The heat treatment may possibly alter the nutritional value of the albumin.

(2) The 25 per cent neutral solution that has been made available to the Armed Forces has the advantage of small volume, high osmotic efficiency, low viscosity and high thermal stability. No changes have been suggested to improve its safety and effectiveness for use in shock.

(3) Use of normal human serum albumin as a diuretic agent has led to the development of a preparation which contains no mercurial preservative and but very little sodium. The clinical studies on the use of such preparations in shock, as well as in other conditions, are reported elsewhere.

(4) One hundred ml. of 25 per cent normal human serum albumin neutralized to pH 6.8 contains 0.2 gram of sodium. It is osmotically equivalent to 450 ml. of plasma which contains 1.6 grams of sodium or to 500 ml. of citrated plasma which contains 2.1 grams. Further reduction in the sodium content of standard neutral albumin preparations could be effected since but 0.1 gram is bound by the albumin and 0.1 gram is present as sodium acetate and citrate.

(5) The addition of 0.1 gram of sodium as the salt of a non-polar anion, such as acetyltryptophanate or mandelate (0.04 molal), is sufficient to render the albumin more stable than in previous preparations which contained 3 times as much sodium.

(6) Acetyltryptophane in this amount is sufficient to supplement the albumin with respect to the amino acid in which it is most deficient. Addition of 0.1 molal isoleucine, in which plasma as a whole is also deficient, renders albumin adequate for the growth of rats.

(7) The standard albumin with 0.04 molal salt is moderately hypotonic. The addition of 0.1 molal amino acid makes it approximately isotonic.

(8) Albumin stabilized with 0.04 molal acetyltryptophane or mandelate may be heated for 10 hours at 60° C. and remain more stable than the present albumin. Still higher temperatures and longer times could be achieved by the use of larger amounts and different combinations of stabilizing reagents. It should thus be possible to determine conditions such that the albumin will remain stable, while bacteria and viruses will be destroyed.

(9) The heating of albumin for 10 hours or more at 60° C. or higher in the final containers

should render it reasonably safe to eliminate the addition of a mercurial bacteriostatic agent and thus to make available a blood derivative of high osmotic efficiency and low sodium content which can be used in large amounts, not only in shock, but in a variety of other conditions.

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THE TREATMENT OF EXPERIMENTAL HYPOPARATHYROIDISM IN DOGS

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In subtotal thyroidectomy in human subjects, a significant amount of the parathyroid tissue is frequently removed, so that some of these patients show the characteristic symptoms of hypoparathyroidism. Although preparations of parathyroid hormone are available for the treatment of this condition, administration of dihydrotachysterol (Hytakerol) has become largely the method of choice, and clinical experience with it has been almost entirely favorable (1 to 3). A review of the chemical and biological properties of this preparation has been published by another investigator (4). Vitamins D_2 and D_3 are calcemic and therefore might also be satisfactory for the treatment of these patients on theoretical grounds. In fact, there are clinical reports which indicate that the substitution may be made (5 to 8). It has been pointed out (9) that the chief bar to such substitution is psychological, *i.e.*, there is a fear of overdosage (since adequate therapy may require millions of units) or of toxicity (since the early preparations of vitamin D_2 did actually contain toxic irradiation by-products). A comparative study of the effects of adequate doses of the 3 preparations on the calcium and phosphorus metabolism of the normal dog has been reported from this laboratory (10).

Since data on human subjects are accumulated very slowly, it seemed worth while to carry out a study of the treatment of parathyroid insufficiency in dogs, comparing the methods of treatment now commonly used; namely, the administration of calcium salts, parathyroid extract, vitamin D_2 , vitamin D_3 , and dihydrotachysterol. The purpose of this work has been (a) to determine the doses of these preparations required to bring dogs from the borderline of tetany to normal or slightly-elevated serum calcium values, (b) to determine the duration of action of therapeutic doses, and (c) to compare the calcium and phosphorus metabolism of normal and thyroid-parathyroidectomized dogs. It was hoped that the principles

which might be developed would facilitate and stimulate further comparative trials of the 3 principles in the treatment of human parathyroid insufficiency.

EXPERIMENTAL METHODS

Ten adult mongrel dogs in good health were selected as experimental subjects. They were taken in 2 groups of 5 in order that the principles discovered experimentally in the first group could be checked in the second group. For a period of several weeks the first group of animals was accustomed to a standard diet consisting of Old Trusty Supreme Meal,¹ 1 per cent of bone meal (to prevent diarrhea) and 1 pint of milk daily (Diet I). The diet was given in adequate amounts for the size of the dogs involved. However, later it was found necessary to change this diet to one having a lower calcium and phosphorus content, but with the same caloric value.

During the preliminary control period a number of determinations of serum calcium and phosphorus were made. The values found fell within the normal range of calcium, 9.5 to 11.2 mgm. per cent, and phosphorus, 3.5 to 5.5 mgm. per cent, except that 1 dog usually had a phosphorus of 7 to 8 mgm. per cent. Calcium was determined (usually 3 times weekly throughout the experiment unless stated otherwise) by the method of Clark and Collip (11), and phosphorus (less frequently) by the method of Fiske and Subbarow (12).

Total thyroid- and parathyroidectomy was performed under aseptic conditions. Calcium determinations were then made daily until such time as the dog went into tetany, or until the serum calcium fell to very low levels (*i.e.*, 7.5 mgm. per cent or less). This usually occurred within 2 or 3 days but in a few cases was delayed as long as 6 or 7 days. When the serum calcium fell to these low levels the animals were considered ready for study.

RESULTS

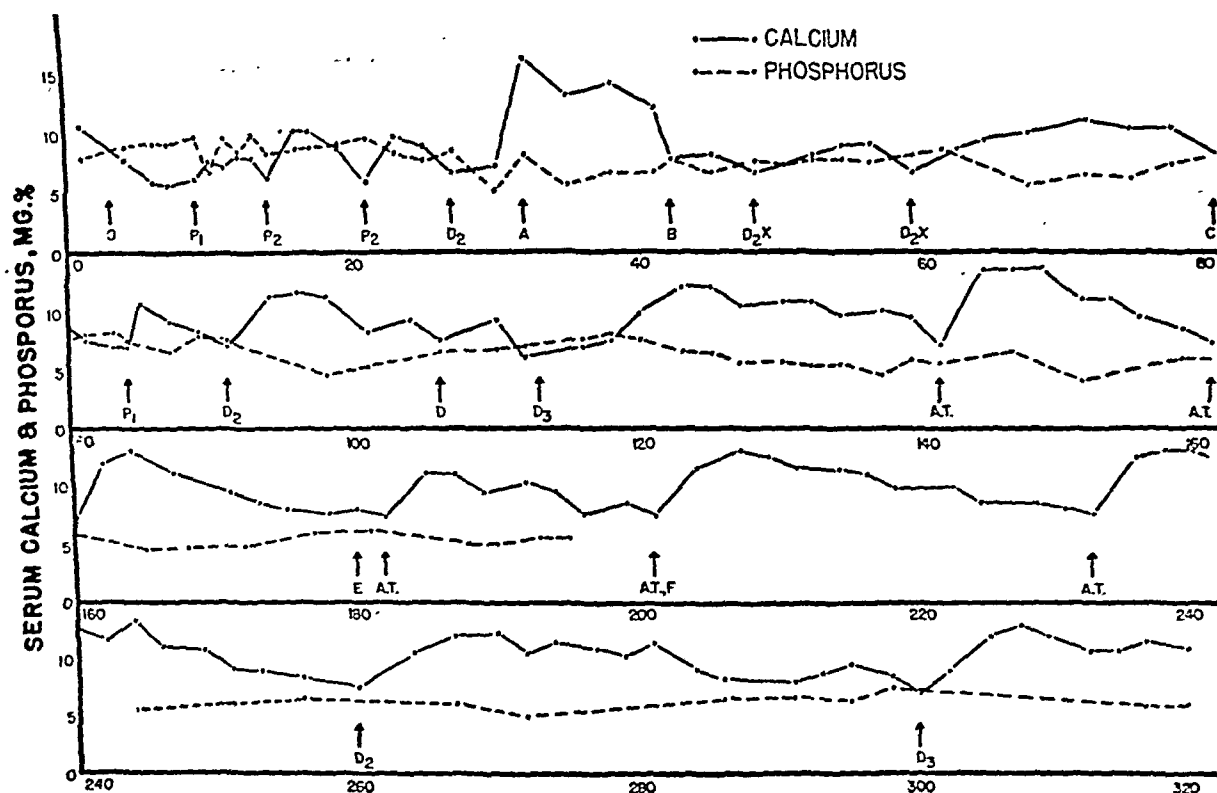
The experimental protocols are too extensive to give in detail. The pertinent data for 1 typical

¹ Manufactured by Old Trusty Dog Food Company, Needham Heights, Mass. Their analysis:

Protein	28 per cent	Fiber	3.5 per cent
Fat	4 per cent	Ash	10 per cent
Carbohydrate	47 per cent	Calcium Phosphorus ratio	1.5/1

Our analysis:

Calcium 1.47 per cent; Phosphorus 0.75 per cent



TIME IN DAYS

FIG. 1. BLOOD SERUM CALCIUM AND INORGANIC PHOSPHORUS OF 1 DOG FOR THE ENTIRE EXPERIMENTAL PERIOD

- O = Operated
- P₁ = Parathyroid extract, 50 units per kgm.
- P₂ = Parathyroid extract, 100 units per kgm.
- D₂ = Vitamin D₂, 2 mgm. per kgm.
- A = Changed to liver diet
- B = Returned to diet I
- D₂X = Vitamin D₂, 0.5 mgm. per kgm.
- C = Changed to liver diet
- D = Changed to diet II
- D₃ = Vitamin D₃, 2 mgm. per kgm.
- A.T. = Dihydratachysterol, 1 mgm. per kgm.
- E = Added calcium carbonate to give calcium/phosphorus: 2/1 in diet
- F = Returned to diet I

animal, for the entire experimental period, are given graphically in Figure 1, and a summary of the data on all of the animals is given in Table I. The following is a general characterization of the results and the principles derived from them.

1. *Treatment with calcium gluconate.* A suspension of calcium gluconate in water was given orally in quantities sufficient to represent 1 or 2 grams per kgm. body weight per day (1 dose per day). In addition, the animals were given 1 or

2 grams of ammonium chloride in the form of enteric-coated tablets for the purpose of relieving their alkalosis (13). In this way, it was possible to keep them alive and clinically in good condition, although the serum calcium was never found to rise over 7 mgm. per cent. In the few cases where the animals actually went into tetany, calcium gluconate was injected intravenously as a 5 per cent solution, and afterward oral calcium gluconate administration sufficed.

TABLE I

Effect of various medications and diets on the response of the parathyroidectomized dog

Medication	Units	Average serum calcium			Duration of response	Diet**
		Initial	Peak	Increase		
	mgm. per kgm.				days*	
Parathyroid extract	50	7.0	8.8	1.8	11	I
	50	6.0	9.1	3.1	7	Liver
	50	7.4	11.0	3.6	9	III
	100	6.5	9.6	3.1	10	I
	100	7.3	11.4	4.1	7	III
Vitamin D ₂	5	7.2	17.4	10.2	60	I, II
	2	6.7	16.4	9.7	31	I
	2	7.2	12.3	5.1	24	II
	2	7.8	12.4	4.6	28	III
Vitamin D ₃	2	7.2	12.2	5.0	26	II
	2	7.5	12.9	5.4	27	III
Dihydro-tachysterol	1	7.5	12.8	5.3	41	II
	1	6.8	14.0	7.2	33	III

* Defined as the elapsed time until the serum calcium again fell below 8 mgm. per cent.

** The composition of the diets is stated in the text. The analysis follows: Diet I, 2.40 per cent calcium, 1.15 per cent phosphorus; Diet II, 0.35 per cent calcium, 0.48 per cent phosphorus; Diet III, 0.96 per cent calcium, 0.55 per cent phosphorus. Liver contains about 0.02 per cent calcium, 0.53 per cent phosphorus (variable).

2. *Treatment with parathyroid extract.* The extract was injected as single doses intramuscularly at 2 different levels: 50 and 100 units per kgm. body weight. The response to this treatment was somewhat variable both as to magnitude and duration of rise in serum calcium. In general a dose of 100 units per kgm. caused a maximal rise of 3 to 4 mgm. per cent in serum calcium and a fall of 1 to 3 mgm. per cent in serum phosphorus. The rise in calcium was usually noted within 24 hours, and the maximal rise was ordinarily noted then, although in some cases higher values were obtained on the third or fourth day post-medication. The elevated calcium values persisted for about 7 days as a rule. This is in marked contrast to the response of normal dogs in which an elevated serum calcium persists for only 18 to 36 hours post-medication (14, 15). The response was not noticeably affected by diet, and the difference between the responses to dosages of 50 and 100 units per kgm. was rather small.

The animal considered to be the most typical responded as follows: After a dose of 50 units per kgm. body weight, the serum calcium rose on the third day to a level of 1.8 mgm. per cent above its base line (6.3 mgm.

per cent). The serum phosphorus fell 2.8 mgm. per cent on the first day after medication, but rose again to its base line (9.7 mgm. per cent) on the second day. On the fifth day, both calcium and phosphorus values were at their original levels, and a dose of 100 units per kgm. was given. On the second day after this dose, the serum calcium had increased by 3.9 mgm. per cent. No change in serum phosphorus was noted. The base level for calcium was reached on the seventh day, when another dose of 100 units per kgm. was given. The maximal rise was again 3.9 mgm. per cent on the second day with the base level reached on the seventh day. There was a slight temporary fall in serum phosphorus.

3. *Treatment with vitamin D₂.* The general principles we followed in studying the activated sterols were: (1) to determine the amount of the single dose required to elevate the serum calcium from a value of 6 to 8 mgm. per cent to 11 to 12 mgm. per cent, and (2) to study the duration of the effects of single doses so chosen.

It was found earlier (10) that doses of 5 mgm. per kgm. of vitamin D₂ (that is, 200,000 I.U. per kgm.) would cause a rise of 4 to 6 mgm. per cent in the serum calcium of a normal dog. Accordingly, this dosage was given orally in oil to the parathyroidectomized dogs. It proved to be far too much; the serum calcium of the dogs so treated rose rapidly to 16 to 18 mgm. per cent and remained at that level for several days. A more physiological level was obtained by changing the diet. The milk was removed from the diet, and then the bone meal. Finally, it was necessary to remove the Old Trusty Meal and substitute liver, a substance containing phosphorus, but practically devoid of calcium. This brought the calcium down to 8 to 9 mgm. per cent, but any substitution of the original ingredients of the diet produced an immediate rise. A physiological serum calcium value could only be maintained on the very low calcium diet. Subsequently on other animals it was found that a dose of 2 mgm. per kgm., with the original diet, still gave too great a response. However, a dose of 0.75 to 1 mgm. per kgm. gave a rise of 4 to 5 mgm. per cent, which was of the desired magnitude.

In later experiments, a diet (Diet II) consisting of (for a 10-kgm. dog), raw beef liver, 417 grams, Old Trusty Meal, 82 grams, and bone meal, 1.7 grams, was given. This diet contained 0.35 per cent calcium and 0.48 per cent phosphorus. For one period of about a month, it was necessary to substitute pork kidney for beef liver; this did not alter the calcium and phosphorus content of the whole diet appreciably. The diet yielded, for a 10-kgm. dog, 1.75 grams of calcium, 2.40 grams of phosphorus per day. In general, it was completely consumed.

On this diet the dogs at least maintained their body weights and some gained rapidly. A parathyroidectomized dog receiving this diet, and a single dose of 2 mgm. per kgm. of vitamin D₂ (when its serum calcium

was 7 to 8 mgm. per cent), responded with a maximal rise to 11.5 to 13.5 mgm. per cent, usually on the third or fourth day after medication, but occasionally later, and did not need another treatment for 3 to 6 weeks. The average protection was about 35 days.

4. *Treatment with vitamin D₃*. When the work with vitamin D₃ was begun, most of experimental difficulties had been corrected, and it was possible to proceed more directly to the final conclusion. The animals were continued on Diet II and a single dose of 2 mgm. per kgm. was invariably given. The responses very closely paralleled those observed for vitamin D₂. The maximal rise noted was to a level of 11.7 to 13.7 mgm. per cent at from 3 to 7 days post-medication. A rise of this magnitude would require a dosage of 5 mgm. per kgm. in the normal dog. The duration of the response was usually 23 to 30 days, or not particularly different from what was observed (10) in the normal dog. (It was considered that the response was at an end when the serum calcium again fell below 8 mgm. per cent.) The general picture with vitamin D₃ does not differ significantly from that observed with vitamin D₂.

5. *Treatment with dihydrotachysterol*. The bioassay of this material on the basis of its hypercalcemic effect was described in an earlier paper (16). Of the commercial material, 1 ml. contains about 1.25 mgm. of the active principle, and this principle has about twice the calcemic effect of vitamins D₂ or D₃ in the rat (9, 17) but more than twice the calcemic effect of vitamins D₂ and D₃ in the normal dog (10). In human hypoparathyroid subjects, the ratio seems to be about 6:1 since between 4 and 8 mgm. of vitamin D₂ are equivalent to each mgm. of dihydrotachysterol (8, 9). As a first approximation, it was decided to give the dogs 1 mgm. per kgm. of dihydrotachysterol in conjunction with the same diet (II) as used for the vitamin D₂ experiments. The serum calcium rose to the desired levels; the maxima were 11.4 to 14.6 mgm. per cent (usually about 13.0 mgm. per cent) on the third to fifth day post-medication, and the duration of the response was usually 16 to 40 days. Therefore, other dose levels were not investigated.

One animal received 5 successive treatments with this preparation (see Figure 1). The initial serum calcium values were always 7.4 to 7.8 mgm. per cent, and the peak values were 11.4 to 13.9

mgm. per cent. The duration of the response was 19 to 30 days. A radical increase in the calcium content of the diet (from 0.38 per cent calcium, 0.46 per cent phosphorus, to 0.92 per cent calcium, 0.46 per cent phosphorus) did not alter the character of the response either as to magnitude or duration.

REPETITION OF EXPERIMENTS

In order that the conclusions could be verified under better controlled conditions, and profiting by the principles already developed, a second group of dogs was prepared for experiment. They were kept from the outset on a diet (Diet III) containing (for a 10-kgm. dog) Old Trusty Meal, 98 grams, dried bread crumbs, 125 grams, bone meal, 2 grams. This was mixed with water to form a mixture of suitable consistency, and in general it was completely consumed. The dogs all maintained body weight on this diet and some showed considerable gain. The diet analyzed 0.96 per cent calcium, 0.55 per cent phosphorus, and yielded, for a 10-kgm. dog, 2.15 grams calcium and 1.23 grams phosphorus per day.

After the normal serum calcium and phosphorus values had been determined, the reaction of the dogs (before operation) to the dosages of the preparations to be given later was determined. Three dogs received 2 mgm. of vitamin D₂ per kgm. body weight, and their maximal rise in serum calcium averaged 2.2 mgm. per cent (range, 0.8 to 4.5 mgm. per cent). Five dogs received 2 mgm. per kgm. body weight of vitamin D₃, and their average maximal rise in serum calcium was 1.3 mgm. per cent (range, 0 to 3.4 mgm. per cent). Five dogs received 1 mgm. per kgm. body weight of dihydrotachysterol, and their average maximal rise in serum calcium was 4.5 mgm. per cent (range, 2.2 to 6.3 mgm. per cent). Serum phosphorus changes were not striking with any of the 3 products.

After the thyroid-parathyroidectomy, the animals were followed as before at daily intervals until the serum calcium fell below 8 mgm. per cent. Each dog then received a dosage of 50 units parathyroid extract per kgm. body weight, followed by a second dosage of 100 units per kgm. as soon as a calcium level below 8 mgm. per cent had been reached after the first. The maximal rise obtained (3.5 mgm. per cent) and the duration of the response (about 7 days) were practically the same for both dosages.

The dogs were then treated successively with vitamin D₂, vitamin D₃, and dihydrotachysterol in the dosages previously used. The serum calcium in each case rose from about 7.5 mgm. per cent to a level of 12.5 to 14.5 mgm. per cent on the third to seventh day post-medication.

tion, and did not fall below 8 mgm. per cent again for 25 to 35 days. In general the dihydrotachysterol caused greater rises in serum calcium than did the other 2 preparations, suggesting that, if anything, it is somewhat more than twice as active hypercalcemically in the dog.

DISCUSSION

The manner in which parathyroid extract and the activated sterols bring about a rise in serum calcium has been the subject of considerable investigation. The generally accepted hypothesis (18 to 20) which has been supported by other workers (21) is based on the reciprocal relationship of serum calcium and phosphorus and supposes that the various principles act either by increasing the renal excretion of phosphate or intestinal absorption of calcium. A preparation such as parathyroid extract, which was believed to act ultimately by increasing renal excretion of phosphate, was supposed to increase serum calcium chiefly by mobilization of bone salt. Vitamin D was believed to act ultimately by increasing intestinal absorption of calcium, and dihydrotachysterol appeared to occupy an intermediate position.

Recent work (22 to 24) has tended to show that this hypothesis, insofar as parathyroid hormone is concerned, is not entirely adequate. The hormone is capable of causing a solution of bone salt even in nephrectomized animals. No evidence has been brought forward to show that the hypothesis outlined is incorrect as to the mode of action of the activated sterols.

It is felt that the present work has some bearing on this problem. Thus, it has been shown that vitamin D has a considerably greater calcemic action in the thyroid-parathyroidectomized dog than in the normal animal. (This is in agreement with the findings of a group of workers on human subjects (25).) It must be kept in mind, however, that in these animals the thyroid glands were completely removed so that the dogs suffered from a thyroid deficiency, not usually observed in the human patients. This action depends on the calcium content of the diet. On a diet of average calcium content (for a normal dog), a dose of 5 mgm. per kgm. of vitamin D₂ produces an alarming rise (about 10 mgm. per cent) in the serum calcium of the parathyroidectomized dog. By the simple expedient of removing practically all of the calcium of the diet, the serum calcium may be

brought down to physiological levels (or even lower), and may be caused to rise rapidly again by restoring the calcium to the diet. These observations suggest that in the parathyroidectomized dog the elevation of serum calcium is accomplished, following vitamin D, by increased absorption from the gut. However, the animal lacks the ability to stop, or otherwise compensate for (by increased excretion or bone formation), the absorption process once the normal calcium level is reached. A normal dog, receiving the same diet and vitamin dosage, shows a more moderate rise in serum calcium (4 to 5 mgm. per cent), indicating that it has a greater ability to regulate serum calcium at physiological levels in spite of the increased absorption which is known to occur.

The present theories of the action of vitamin D and of the parathyroid gland do not appear to explain satisfactorily why vitamin D should produce a greater calcemic effect in the parathyroidectomized dog than in the normal dog. Furthermore, the administration of parathyroid extract to the parathyroidectomized dog does not cause it to react to vitamin D like a normal animal. We have found that parathyroidectomized dogs which have a serum calcium of 8 mgm. per cent, and which receive a dose of vitamin D₂ (2 mgm. per kgm.) usually sufficient to raise the serum calcium to 11 to 12 mgm. per cent, but which also receive simultaneously 100 units per kgm. of parathyroid extract, respond with a peak serum calcium value of 15 to 18 mgm. per cent. The parathyroid extract, instead of diminishing the effect of vitamin D, has an additive effect. This suggests that the intact parathyroid gland is in some manner able to react positively against elevated serum calcium (perhaps by decreasing the renal threshold for calcium), and that this antagonistic principle is not contained in the parathyroid extracts prepared according to Collip and Clark.

As is well known, parathyroid extract has been found to raise serum calcium while decreasing serum phosphorus. On the other hand, a massive dose of vitamin D in the parathyroidectomized dog causes a rise in *both* calcium and phosphorus. We observed in 1 animal a serum calcium of 16 and a phosphorus of 10 following an overdose of vitamin D₂.

Dihydrotachysterol produces much the same responses in the parathyroidectomized dog as it does in the normal dog. Thus, in the normal dogs a dosage of 1 mgm. per kgm. causes an average rise of 4.5 mgm. per cent. Postoperatively in the same dogs this dosage caused an average rise of 7.7 mgm. per cent. Similarly, a dosage of vitamin D₂ which caused an average rise of 2.2 mgm. per cent normally caused an average rise of 5 mgm. per cent postoperatively, and for vitamin D₃ the corresponding figures were 1.3 and 5.7 mgm. per cent. The differences among the 3 preparations are not great but are consistent with the view that dihydrotachysterol does not act in exactly the same manner. The experiment in which the calcium content of the diet was radically increased without affecting the response to dihydrotachysterol supports the same view. Two investigators (26) have concluded that the administration of dihydrotachysterol causes: (1) an increase in urinary phosphate excretion with the serum phosphorus becoming normal, (2) an increase in the absorption of calcium with higher serum calcium, and (3) no increased output of phosphate in advanced renal damage.

The 3 activated sterols were equally suitable for the management of parathyroid insufficiency in the dog so far as the criteria used in these experiments could distinguish. Where it is possible to judge from other symptoms, as in human subjects, the same would not necessarily apply (8).

SUMMARY

A series of thyroid-parathyroidectomized dogs has been treated with calcium salts, parathyroid extract, vitamins D₂ and D₃, and dihydrotachysterol. With calcium salts, tetany could be avoided, but the serum calcium levels remained very low. Parathyroid extract in dosages of 50 or 100 units per kgm. elevated the serum calcium for periods averaging about 5 days. Normal serum calcium levels (above 9 mgm. per cent) prevailed for about 2 to 3 days of this time.

On a diet of medium calcium and phosphorus content, a single dose of 2 mgm. per kgm. of either vitamin D₂ or D₃ produced a rise in serum calcium of about the desired magnitude (from a base level of 7 to 8 mgm. per cent to a peak value of 11 to 12 mgm. per cent), and the effect lasted

(i.e., the serum calcium did not fall below 8 mgm. per cent) for 25 to 35 days. There was no significant difference in the duration of the effects of the 2 vitamins. It was possible to produce an effect of about the same duration and of slightly greater magnitude by giving 1 mgm. per kgm. of dihydrotachysterol. The quantities mentioned appear to be equivalent for the parathyroidectomized dog.

The possible bearing of the observations on the mechanism of the action of the activated sterols is discussed.

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ELECTROCARDIOGRAMS IN TRAUMATIC SHOCK IN MAN

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As traumatic shock develops, cardiac output falls. There are several fairly well-established explanations for this. It is usually stated, however, that this diminishing output is due to phenomena arising outside of the heart and that the heart itself remains unimpaired until very late. We had hoped to obtain evidence that would, insofar as could be judged by electrocardiograms, give information clearly for or against this view. It was thought likely that as a result of the inadequate circulating blood volume, the lowered blood pressure, and the compensatory tachycardia, the coronary circulation might suffer to an extent recognizable by electrocardiogram.

We are recording herewith observations on a series of 58 electrocardiograms obtained on 30 seriously-wounded soldiers in traumatic shock. The tracings were assembled at the 94th Evacuation Hospital in February and March of 1944, during the operations on the Cassino Front, and

subsequently in April and May, on the Anzio Beachhead. When possible, tracings were made both during shock and in the recovery phase. In 10 patients (one-third of the group), the blood pressure could not be measured on entry. In the remaining two-thirds the degree of circulatory collapse was somewhat less severe as reflected by blood pressures of 60 to 70 mm. Hg systolic and 20 to 40 mm. Hg diastolic. Blood pressure determinations were made with a mercury manometer or with aneroid manometers checked against the mercury.

THE ELECTROCARDIOGRAMS

Definite abnormalities of the electrocardiograms were encountered in 5 of the 30 patients studied, but the most striking feature was the normal character of most of the tracings (in 25 of the 30 patients). These normal records were observed in many of those in extreme shock as well as in those

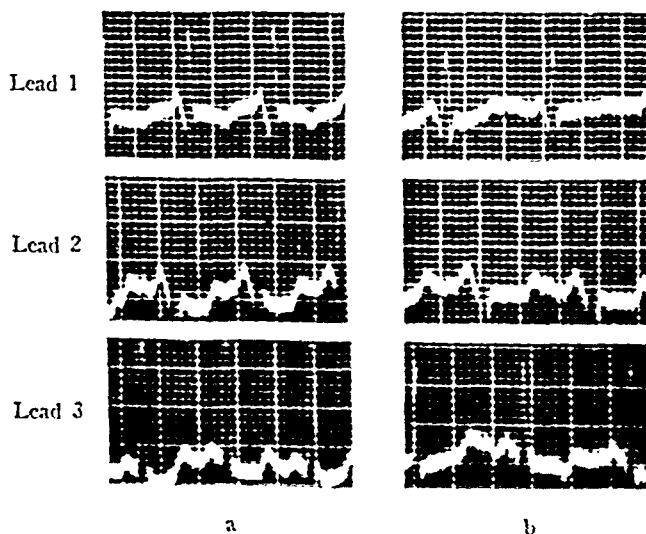


FIG. 1. ELECTROCARDIOGRAMS—CASE 1

a. Showing late inversion of the T waves in lead 1, and diphasic T waves in leads 2 and 3.

b. From the same patient taken 30 minutes later after the shock had been relieved. It shows a striking improvement in the T waves, especially in lead 1.

in better condition. However, in the 5 patients where abnormalities were recorded, they are of sufficient interest to warrant a brief discussion.

Abnormal T waves. A definite but transient inversion of the T wave in lead 1 was observed in 2 instances.

Case 1. J. B. C., age 27, received a severe wound of the left chest with open pneumothorax. He was brought to the hospital within a few hours after emergency treatment in the field. On arrival he was in profound shock with unmeasurable blood pressure (hemoglobin 13 grams, hematocrit 38). The electrocardiogram taken at this time showed a sharp late inversion of T 1 and moderate depression of the S-T interval in leads 2 and 3 (Figure 1a). Following administration of about a liter of whole blood in a half hour, his circulatory state improved promptly and his blood pressure had risen from 0 to 125 mm. Hg systolic and 65 mm. Hg diastolic. A second electrocardiogram (Figure 1b) taken 30 minutes after the first showed a remarkable improvement in T 1 to a low but upright position, and a lessening in the diphasic contour of S-T 2 and 3. There was no evidence of cardiac or pericardial injury in this case, and the prompt return of the inverted T 1 towards normal coincident with improvement in the general circulation suggests that mechanical trauma of the heart was not a factor in this temporary abnormality. Subsequent to operation the clinical course insofar as known was uneventful.

Case 2. A. H., age 45, received a penetrating wound of the left chest. Upon arrival at the hospital a few hours later, he was in severe shock. His blood pressure

was 55 mm. Hg systolic and the diastolic level was undetectable (hemoglobin 11.5 grams, hematocrit 34). An electrocardiogram (Figure 2a) revealed a sharp late inversion of the T waves in lead 1 without other abnormality. A superimposed artefact due to alternating current is well displayed in this and the subsequent tracing. It was a frequent complication in the tracings taken on the Anzio Beachhead and at times difficult to eliminate. Following administration of 1 unit of albumin and 500 ml. whole blood, the patient responded satisfactorily. At the end of an hour, his blood pressure had risen to 90 mm. Hg systolic and 60 mm. Hg diastolic. An electrocardiogram (Figure 2b) showed a return to normal of the previously inverted T 1. There was again no evidence of lasting cardiac trauma and the subsequent course was uneventful.

Abnormal axis deviation (temporary). In planning this study we had anticipated encountering electrocardiographic evidence of temporary strain upon the right ventricle (right axis deviation) as a result of liberal use of intravenous fluids (plasma and blood) in combatting peripheral circulatory collapse. The following case is the only instance in which right axis deviation was noted.

Case 3. H. G. K., age (c.) 25, received a penetrating wound of the right chest with pneumothorax and retained foreign body. Upon arrival at the hospital he was in moderate circulatory collapse (hemoglobin 11.2 grams, hematocrit 33). His blood pressure was 65 mm. Hg

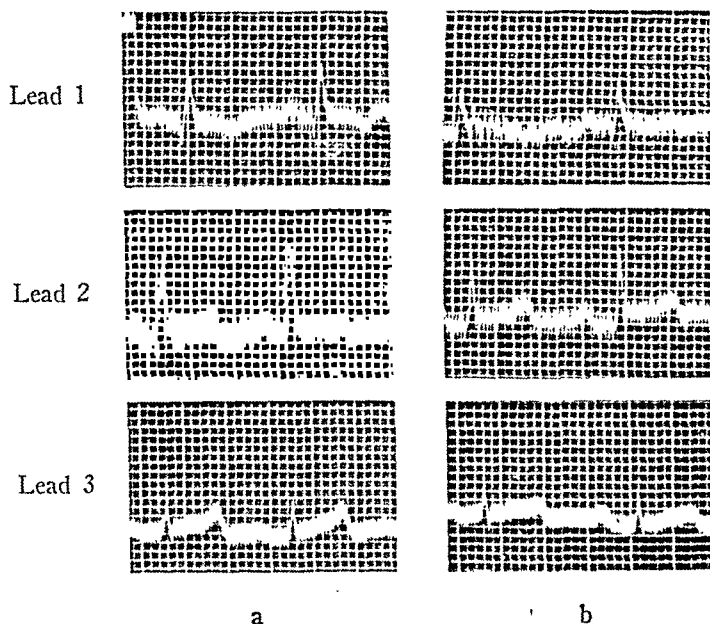


FIG. 2. ELECTROCARDIOGRAMS—CASE 2

a. In traumatic shock, showing late inversion of the T waves in lead 1.

b. From the same patient 1 hour later showing improvement in the T waves coincident with relief of circulatory collapse.

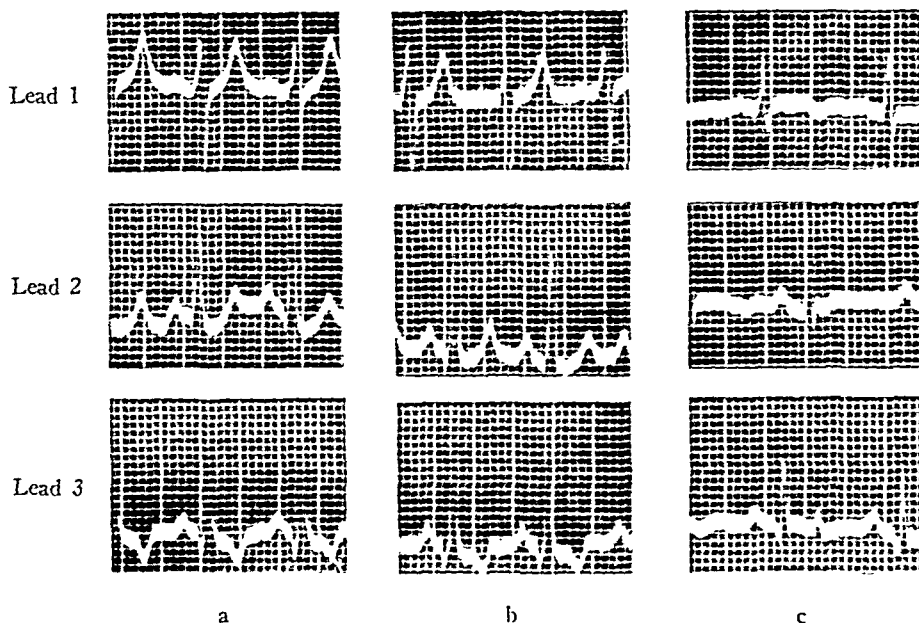


FIG. 3. ELECTROCARDIOGRAMS—CASE 3

- a. Showing well marked right axis deviation.
- b. After 30 minutes showing no change.
- c. Following operation and 24 hours after the first tracing. It now shows normal axis deviation.

systolic and 20 mm. Hg diastolic, and an electrocardiogram (Figure 3a) showed well-marked right axis deviation. There was no evidence in the liver or in the peripheral veins of right-sided heart failure. At the end of 30 minutes his circulatory state had deteriorated, in that his systolic blood pressure had dropped to 40 mm. Hg and the diastolic level was unobtainable. An electrocardiogram at this time showed no change (Figure 3b). He received 1000 ml. whole blood and 500 ml. of 2 per cent sodium bicarbonate solution intravenously in $2\frac{1}{2}$ hours preceding operation without signs of heart failure. The chest wound was successfully closed, and 18 hours after operation (and 24 hours after the original tracing) his condition was good. The blood pressure had returned to 110 mm. Hg systolic and 70 mm. Hg diastolic. The electrocardiogram showed a striking shift to normal of the axis deviation and concurrent changes in the contour of the T waves, the latter probably of no special significance. In the absence of other evidence of cardiac injury or strain, and the normal response of the heart to the intravenous infusion, we are inclined to interpret the changing electrocardiographic pattern in this patient as most likely of mechanical origin related to displacement (torsion) of the heart by blood, air, or injured lung prior to operation.

Miscellaneous abnormalities, including abnormal QRS complexes and cardiac irritability.

Case 4. L. K. R., age (c.) 25, received a penetrating wound of the left chest, perforation and hematoma of the left lung (lower lobe), laceration of the diaphragm and

spleen, and multiple injuries of the extremities. Splenectomy was necessary shortly after arrival in the hospital. Post-operatively his condition was poor; in spite of supportive therapy, his blood pressure was 80 mm. Hg systolic and 40 mm. Hg diastolic. An electrocardiogram taken at this time (2 hours before death) (Figure 4) showed abnormal W-shaped QRS complexes of low voltage, the explanation for which is not clear, but is probably related to extensive trauma in close proximity to the heart, possibly of the heart or pericardium.

Case 5. J. R. P., age 20, was wounded severely in the buttocks, genitals, and extremities. He was in extreme shock with unmeasurable blood pressure (hemoglobin 8.5 grams, hematocrit 25). Four hours after he was wounded, and 2 hours after he was hospitalized, he developed marked cardiac irritability, manifested by paroxysmal auricular fibrillation with a ventricular rate of 180 with short runs of ventricular tachycardia superimposed. Despite 500 ml. of plasma, 1750 ml. of whole blood and 500 ml. of 2 per cent sodium bicarbonate solution intravenously, his blood pressure had risen only to 78 mm. Hg systolic and 50 mm. Hg diastolic after 3 hours, and surgery was undertaken, the auricular fibrillation persisting. Following operation, he made a satisfactory recovery.

DISCUSSION

The abnormalities in the electrocardiograms recorded above are of some interest, and difficult to explain. In no instance did we observe clinical

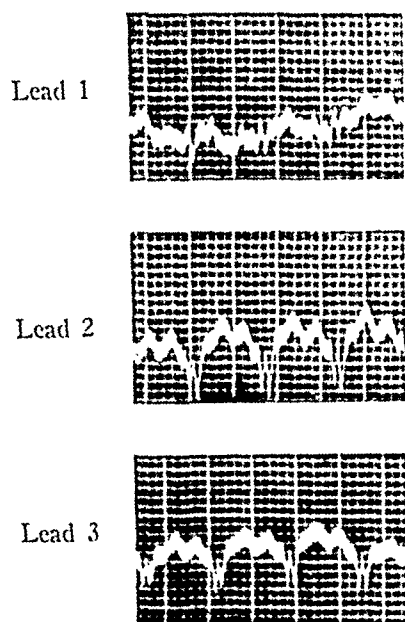


FIG. 4. ELECTROCARDIOGRAM (CASE 4) SHOWING BIZARRE QRS COMPLEXES OF LOW VOLTAGE

signs of cardiac weakness in the form of abnormal accentuation of the pulmonary second sound, basal râles, gallop rhythm, or congestion of the cervical veins or of the liver. As remarked above, the majority of the electrocardiograms were normal in character. Several patients in the series were in severe shock with low blood pressures for a period of hours with no effect upon the electrocardio-

gram. It may be significant that in both patients with the transient inversion of the T waves in lead 1 the wound involved the left chest, although as far as could be determined by x-ray and at the time of operation, the heart and pericardium escaped injury. Furthermore, the transient nature of the inversion was more in accord with a temporary functional disturbance (possibly hypoxia) than with lasting tissue injury.

SUMMARY

From a study of 58 electrocardiograms on 30 severely wounded soldiers in traumatic shock it was found that: (1) the majority were normal; (2) in 2 instances striking, but transient, inversion of the T wave in lead 1 was noted; (3) in 1 instance, complicated by intrathoracic injury, there was a shift from marked right axis deviation back to normal following operation; (4) another patient showed bizarre QRS complexes of low voltage, and a fifth patient showed an unusual degree of temporary cardiac irritability with paroxysmal fibrillation and ventricular tachycardia. The possible significance of these changes has been discussed.

We are indebted to The Surgeon, Fifth Army, and to the Commanding Officer, 94th Evacuation Hospital, for their authorization of and co-operation in this study.

QUANTITATIVE ELECTROENCEPHALOGRAPHIC STUDIES OF ANOXIA IN HUMANS; COMPARISON WITH ACUTE ALCOHOLIC INTOXICATION AND HYPOGLYCEMIA¹

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That disturbances in consciousness, and consequently in behavior, are produced by anoxia is well known. The clinical picture is essentially that of delirium, a syndrome in which we have pointed out that the primary psychologic symptom is a reduction in the level of consciousness (1). With moderate or severe degree of anoxia the changes are grossly obvious.

The evaluation of the harmful effects of mild degrees of anoxia, however, has proven extremely difficult due to the lack of accurate methods of measuring such effects. Such measurements are of obvious importance to aviation medicine and are of interest in studies of the earliest changes in delirium. So far, the most satisfactory methods have concerned changes in visual functions. Various performance and psychological tests have all proven relatively unsatisfactory due to the wide range of individual responses, doubtless influenced by motivation and emotional factors. In such studies statistical analyses of a large number of subjects indicate that significant degrees of impairment of performance begin around 12,000 feet (483.3 mm. Hg).

Numerous investigators have reported the effects of anoxia, hypoglycemia, and various drugs on the electroencephalogram, but for the most part, these studies have been concerned with the more marked changes. The demonstration of a close relationship between changes in frequency distribution in the electroencephalogram and levels of consciousness (1) and the development of a quantitative method for analyzing electroencephalographic frequency spectra (2) offer a practical approach to the study of cortical function during mild to moderate degrees of anoxia. The

study of the normal electroencephalogram with this method reveals a continuous range of frequency change during variations within the physiological zone of blood sugar, oxygen, and acid-base balance. Contrary to prevailing notions, the transition between this physiological zone and a pathological zone is subtle and not abrupt. Studies with hypoglycemia, alcohol, and other toxic materials have clearly demonstrated that the degree of change in frequency is of far more importance than the appearance of any particular wave frequency (*i.e.*, delta waves). For example, equivalent degrees of acute alcoholic intoxication with comparable reduction in the level of consciousness in different individuals may be associated with widely different frequency distribution, but the degree of change in frequency from the control record is always comparable for all subjects (3). These data clearly demonstrate the inadequacy of the crude methods of interpretation of the electroencephalogram which depend upon the appearance of abnormally slow waves (2 to 7 per second), and explain why previous brain wave studies have not proven fruitful in the evaluation of intermediary zones of acute anoxia, where gross changes in the level of consciousness are not usually apparent.

METHODS AND MATERIAL

Seven medical students and 3 staff members acted as subjects for the experiments. They were exposed in the decompression chamber to simulated altitudes of 10,000 to 16,000 feet breathing air, and 30,000 to 42,500 feet breathing 100 per cent oxygen for varying periods of time up to 3 hours. A Bulbular type 14 demand mask modified for constant flow, was used. When several altitudes were tested on the same flight, possible cumulative effects were avoided by having the subjects breathe at a higher than normal oxygen tension for 10 minutes before starting the next period of anoxia. Blood sugar levels were obtained at frequent intervals since the frequency spectra vary with changes in blood sugar (2). Blood sugar was determined by the Folin-Wu method.

¹ The work described in this paper was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Cincinnati.

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The effect of exposure to altitudes of 10,000 to 16,000 feet on the E.E.G. frequency spectra
This illustrates the magnitude of the spectral shift and the consistency with which the mean frequency diminished in 4 subjects exposed 11 times.

TABLE I

TABLE I														FERRIS	
Altitudes of 10,000 to 16,000 feet on the E.E.G. frequency spectra														Consistency with which the mean frequency diminished	
Name and date	Altitude		Time minutes	E.E.G. frequency spectra							E.E.G. mean freq.	Arm vein blood sugar			
	feet	mm. Hg		LVF*	12	11	10	9	8	7			6		
EF 11-30-44	Ground 10,000 14,000 16,000 Ground	747 523 446 412 747	30 30 30 5												
EF (1) 10-14-44	Ground 10,000 10,000 Ground	747 523 523 747	5 60 5												
EF (2) 10-15-44	Ground 10,000 12,000 12,000 Ground	747 523 483 483 747	5 60 5												
EF (3) 10-20-44	Ground 10,000 12,000 14,000 14,000 Ground	747 523 483 446 446 747	5 60 5												
EF (4) 11-12-44	Ground 14,000 14,000 14,000 14,000 Ground	747 446 446 446 446 747	5 30 60 120 180 5												
GE (1) 11-12-44	Ground 14,000 14,000 14,000 14,000 Ground	747 446 446 446 446 747	5 30 60 120 180 5												
GE (3) 10-20-44	Ground 10,000 12,000 14,000 14,000 Ground	747 523 483 446 446 747	5 5 5 60 5												
DS 12-10-44	Ground 10,000 14,000 16,000 Ground	747 523 446 412 747	30 30 30 10												
MR (1) 12-3-44	Ground 10,000 14,000 16,000 Ground	747 523 446 412 747	30 30 30 5												
MR (2) 12-10-44	Ground 10,000 14,000 16,000 Ground	747 523 446 412 747	30 30 30 5												
* Low voltage fast activity.															

* Low voltage fast activity.

metric method, which includes non-glucose reducing substances. We selected subjects whose electroencephalograms showed good alpha rhythm and less than 10 per cent low voltage fast activity, since quantitative analysis of frequency change is far more accurate in such records.

All electroencephalograms consisted of bipolar fronto-occipital tracings. The method of analysis of the electroencephalograms is that described in detail in the report already cited (2). This consisted of counting the number of waves per second interval (utilizing the ruled paper supplied by Mr. Albert Grass) for 200 consecutive seconds. The distribution of frequencies per second was then expressed as a percentage of the whole. A complete wave was one which returned at least two-thirds of the way to the base line. A wave which crossed the line dividing adjacent one-second intervals was counted in the interval containing more than half the wave. Small superimposed waves were not counted. In general, it was found advisable to make full use of the high frequency filter to damp the superimposed fast oscillations of very low voltage (some of which were of muscle origin) so that the basic sine waves would have sharper definition. Stretches of low voltage fast activity were designated as such (LVF) and no attempt was made to count the individual waves, which were often not countable. Such an analysis yields the percentile distribution of waves per second (rather than the distribution of wave lengths). In addition to expressing the result in terms of the distribution of waves per second intervals (frequency spectrum), it was also found of value to calculate the arithmetical mean frequency from this distribution.² This yields a number, the mean of the number of waves per second interval, which allows far more precise comparison of frequency changes under the different conditions than would be possible by comparison of the frequency spectra alone. Since the amount of low voltage fast activity cannot be included in the calculation of mean frequency, the advantage of selecting records with little or no voltage fast activity is obvious.

RESULTS

Table I and Figure 1 illustrate that significant shifts to slower frequencies in the electroencephalographic spectra are demonstrable at 10,000 feet (522.6 mm. Hg) and that this shift becomes more marked with increasing altitudes up to 16,000 feet (411.8 mm. Hg). This shift is not visible on inspection of the record alone. Most of the shift is demonstrable within 5 minutes of reaching altitude and is maximal within 15 to 30 minutes, there being relatively little change thereafter up to 3 hours. The degree of shift at 10,000 feet for 30 to 60 minutes among 4 individuals (6 trials) ranged from 0.25 to 0.47; at 16,000 feet for 30

minutes the degree of shift ranged from 0.65 to 1.35 (4 individuals, 5 flights). In general, the greater shifts occurred in the records with faster control frequencies (Figure 1). Upon return to ground level, the pattern was usually restored to the preflight value within 10 minutes. The factors involved in this lag were not clarified.

Comparative studies on 6 subjects breathing air and breathing 100 per cent oxygen revealed that breathing air at 10,000 feet (522.6 mm. Hg) was equivalent to breathing 100 per cent oxygen at 39,000 feet (147.6 mm. Hg) (see Figure 2) and that breathing air at 16,000 feet (411.8 mm. Hg) was approximately equivalent to breathing 100 per cent oxygen at 42,500 feet (124.8 mm. Hg) (see Figure 3) as regards the degree of shift in the electroencephalographic frequency spectra. Other altitudes were not studied. Again the electroencephalograms with the higher control frequencies tended to show somewhat greater frequency shifts. The development of abdominal distention or of decompression sickness at the higher altitudes did not directly alter the electroencephalogram as had already been noted in earlier studies (4), but it did limit the time at altitude to 10 to 15 minutes so that the frequency changes may not have been fully developed.

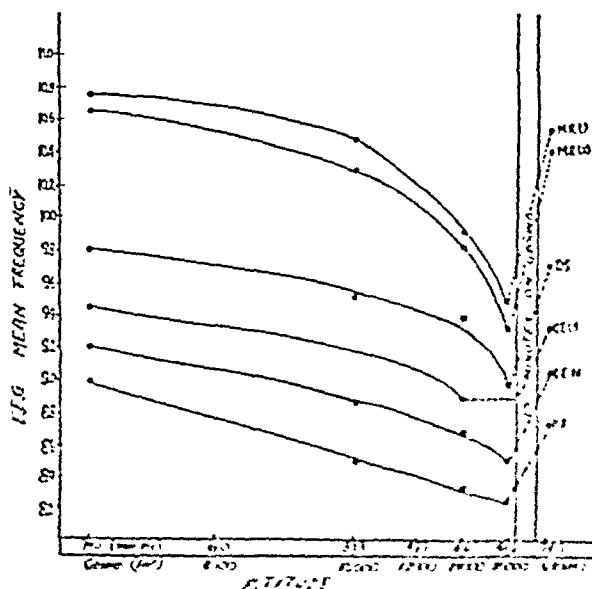


FIG. 1. CHANGES IN ELECTROENCEPHALOGRAPHIC MEAN FREQUENCY WITH DECREASING ATMOSPHERIC PRESSURE (INCREASING ALTITUDE)

² This technique was suggested by Dr. Charles Stevens.

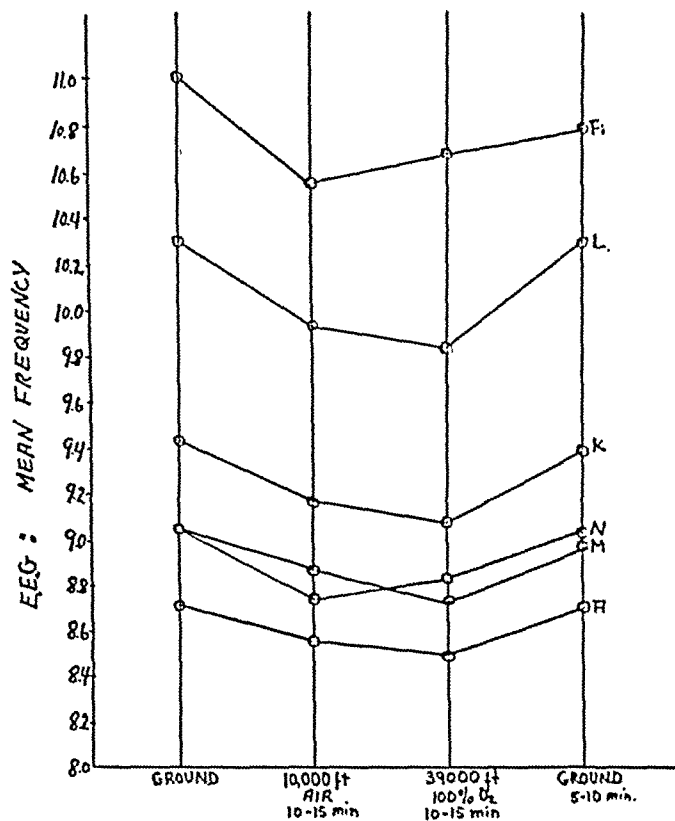


FIG. 2. COMPARATIVE CHANGES IN ELECTROENCEPHALOGRAPHIC MEAN FREQUENCY AT 10,000 FEET BREATHING AIR AND AT 39,000 FEET BREATHING 100 PER CENT OXYGEN

recovery. The results are illustrated in Figures 4 and 5. It was found that the degree of change in the electroencephalogram upon exposure to 16,000 feet (411.8 mm. Hg) was roughly similar to that seen during the period of development of first symptoms of alcoholic intoxication and during reduction of blood sugar to 44 to 49 mgm. per cent. The degree of change was considerably greater during severe intoxication and in the one instance where blood sugar fell to 35 mgm. per cent. Blood sugars of 75 to 80 mgm. per cent appeared to be associated with changes equivalent to 10,000 to 12,000 feet (522.6 to 483.3 mm. Hg).

Certain observations on the behavior of the subjects subjected to anoxia, alcohol and hypoglycemia may be pertinent. All the subjects had made many flights to altitudes of 35,000 to 38,000 feet (178.7 to 154.9 mm. Hg) and although they had had no experience with anoxia, they regarded 16,000 feet with equanimity. At 16,000 feet all the subjects were aware of slight darkening of vision, vague light headedness, and uneasiness. Two subjects, K. and L., became somewhat boi-

Since acute alcoholic intoxication, and to a lesser degree hypoglycemia, are more familiar experiences than anoxia to both physician and layman, it was felt worthwhile to compare with anoxia the electroencephalographic frequency shifts in those conditions in the same subjects. Hypoglycemia was produced in 4 subjects by the intravenous administration of 0.1 units of insulin per kgm. body weight in the fasting state. This reduced blood sugars to the range of 35 to 49 mgm. per cent at which levels the subjects experienced mild hypoglycemic symptoms, with weakness and light-headedness, but no loss of consciousness. On another occasion, acute intoxication was produced in the same subject by the ingestion of 100 ml. of 95 per cent ethyl alcohol. The alcohol was consumed within a 5-minute period in the fasting state. Electroencephalograms were repeated when the subjects noted the first subjective symptoms, which was within 45 to 55 minutes after ingestion. Two subjects, consuming larger amounts of alcohol, were followed through the stage of severe and gross intoxication back to

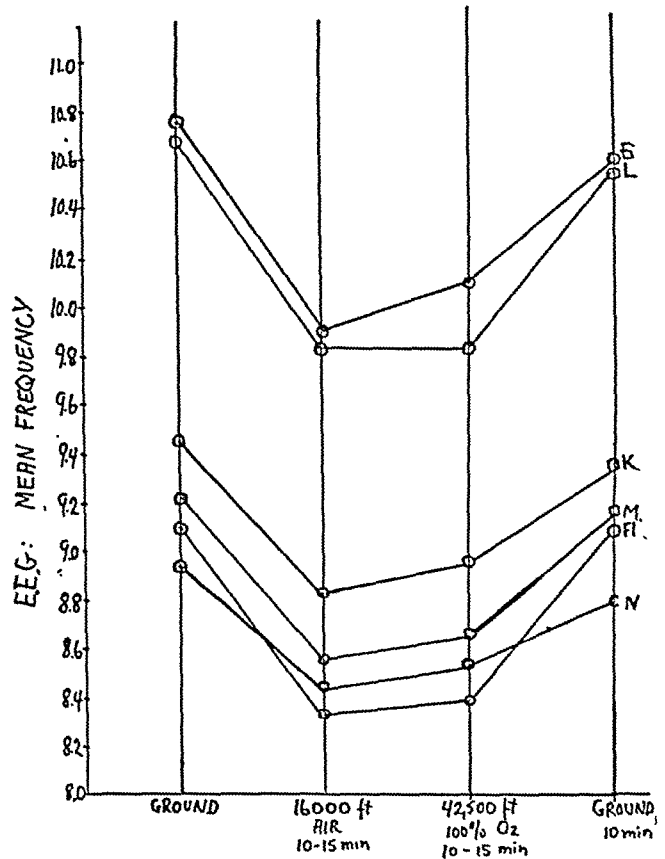


FIG. 3. COMPARATIVE CHANGES IN ELECTROENCEPHALOGRAPHIC MEAN FREQUENCY AT 16,000 FEET BREATHING AIR AND 42,500 FEET BREATHING 100 PER CENT OXYGEN

terous and overtalkative, while M. and N. remained outwardly unperturbed. During the alcohol experiment K. became the caricature of the "happy drunk," a change which began almost before the dose of alcohol had been administered! L. also laughed and joked a great deal, but could be quiet and restrained at will. M. was quiet and unobtrusive, but shortly after the electroencephalogram was taken he developed a reaction of marked withdrawal and bursts of aggressive behavior. N. showed no obvious visible behavior disturbance yet insisted that he felt drunk and would not want to drink any more. During the hypoglycemia experiments, all the subjects became anxious at the time of developing symptoms. The anxiety was most marked in M., least in N. (who had lower blood sugar), and K. and L. in contrast to their behavior during the alcoholic experiment were quiet, anxious, and repeatedly demanded re-

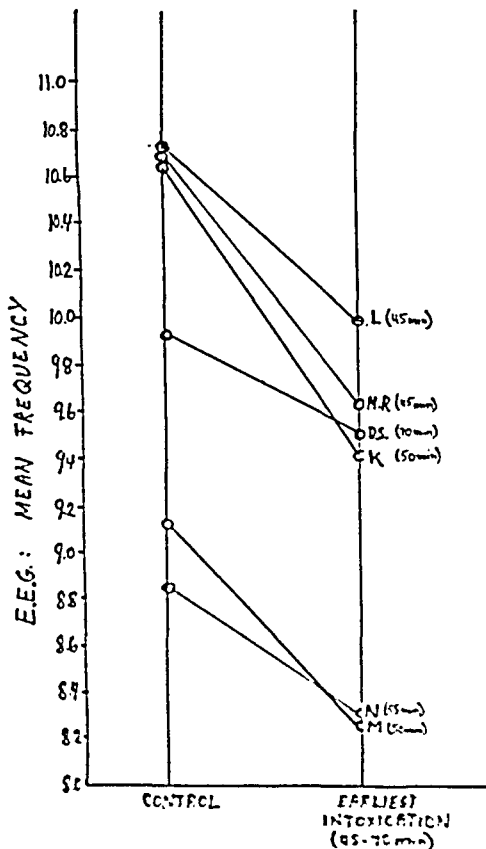


FIG. 4. CHANGES IN ELECTROENCEPHALOGRAPHIC MEAN FREQUENCY OCCURRING DURING EARLIEST SIGNS OF INTOXICATION (SAME SUBJECTS AS IN PREVIOUS FIGURES)

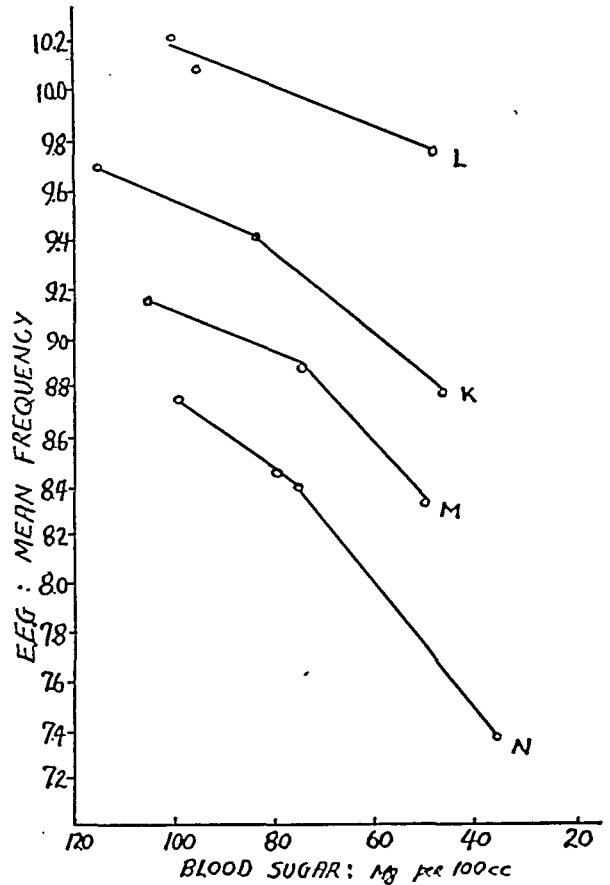


FIG. 5. CHANGES IN ELECTROENCEPHALOGRAPHIC MEAN FREQUENCY DURING REDUCTION OF BLOOD SUGAR TO FROM 36 TO 49 MGM. PER CENT (SAME SUBJECTS AS IN PREVIOUS FIGURES)

assurance as to the outcome of the experiment. In all instances, the degree of disturbance in consciousness, as closely as it could be assayed clinically and by the serial subtraction test was roughly comparable. It is clear from these observations that certain emotional factors played an important rôle in determining the behavior patterns. To these subjects, high altitude exposure was a familiar experience which provoked little anxiety, and was handled with confidence and assurance. During the alcoholic intoxication their behavior was conditioned by certain familiar social patterns and by previous experience. Hypoglycemia, on the other hand, a new and unfamiliar experience to all, provoked more anxiety than any of the other procedures. The reactions during comparable degrees of disturbance in the level of consciousness (relatively mild in these cases) and com-

parable degree of electroencephalographic change are thus dependent both on the previous personality structure of the individual and on the psychologic and emotional significance of the particular experience.

COMMENT

These studies indicate that measurable changes in the electroencephalogram are demonstrable at an altitude of 10,000 feet (522.6 mm. Hg) and increase progressively with the fall in atmospheric pressure. This change is not apparent on inspection alone. At 16,000 feet (411.8 mm. Hg) the degree of change had attained a magnitude comparable to that seen during the earliest phase of acute alcoholic intoxication, and comparable to that seen upon reduction in blood sugar to levels of 40 to 49 mgm. per cent. Such a state is probably incompatible with maximum efficiency, even though some individuals may be able to compensate by heightened effort. It is important, too, that the change is apparent in as short a time as 5 minutes. The degree of change at 10,000 feet (522.6 mm. Hg) was still within the range found among normal individuals during variations of blood sugar from 72 to 140 mgm. per cent and hence must be considered within physiological range. It should be emphasized that these experiments were carried out at complete rest. Obviously, with physical exertion further changes may be anticipated. Further changes may also be anticipated with longer exposures.

These data confirm and extends previous observations on the nature of delirium (1, 3). The primary psychologic symptom of delirium is a decrease in the level of consciousness with increased fluctuation in the level of awareness. In spontaneous delirium occurring during the course of many different clinical conditions (1) and in acute reactions induced by ingestion of alcohol and other toxic materials (3) we have demonstrated close correlation between electroencephalographic changes and alterations in the level of consciousness. In both spontaneous and induced reactions the earliest change in the electroencephalogram is a shift toward slower frequencies with little or no change in regularity or voltage. The degree of anoxia studied in these experiments represents the earliest zone in which changes

would be anticipated and it is obviously not possible to set any sharp line of demarcation, clinically or electroencephalographically, between normal and abnormal. The slowing of the brain waves, and with it the probable alteration in cortical function, is progressive, but individuals still vary widely in their ego strength and in their means of defense. The factors of ego strength, previous personality structure, the setting and meaning of the experience as well as the structural integrity of the nervous system, are obviously most important in these borderline zones, where they make the difference between whether or not the symptoms develop and efficiency is impaired. In more extreme zones the changes in consciousness are readily demonstrable in all people.

These studies also demonstrate the great value of the electroencephalogram as an objective method of studying the effects of noxious influences on the cortex. In different individuals the degree of change in electroencephalographic mean frequency tended to be quite similar under comparable circumstances even though behavior varied considerably among the different individuals and in the different experimental circumstances. The degree of change in the level of consciousness, however, was, in so far as it could be estimated, similar in all subjects. This correlation has been brought out more clearly in studies which concerned more marked disturbances in the level of consciousness (1, 3).

The consistency in the change in the electroencephalographic mean frequency makes possible the evaluation of noxious effects with relatively few subjects and allows for the delineation of safe zones. This is of particular value in problems of military, aviation and industrial medicine where optimum performance is often required in spite of exposure to low oxygen tensions, to noxious gases (carbon monoxide, volatile gases, etc.) or to prophylactic drugs (sulfonamides, antimalarial drugs, etc.). Unpublished studies indicate that stimulant as well as depressant effects may be studied by this technique (3).

SUMMARY

1. Significant changes in frequency distribution of the electroencephalogram are demonstrable at altitudes of 10,000 feet and increase progressively

with further increases in altitude. The changes are present within 5 minutes of reaching altitude, are fully developed within 15 minutes, and thereafter do not increase significantly up to 3 hours. Upon return to the ground, the electroencephalogram usually returns to base line status within 10 minutes.

2. The degree of change for each altitude could be reproduced in each subject on repeated examinations. At each altitude the degree of change was approximately the same for all individuals. The electroencephalograms with higher frequencies tended to show greater frequency shifts under comparable conditions.

3. The degree of frequency change at 39,000 feet breathing 100 per cent oxygen with the constant flow system was the same as that found at 10,000 feet breathing air; 42,500 feet breathing 100 per cent oxygen was the same as 16,000 feet breathing air.

4. Acute alcoholic intoxication of a degree likely to be experienced by most healthy individuals drinking socially provoked electroencephalographic changes roughly equivalent to those found at 16,000 feet. Severe intoxication produced more marked changes.

5. Reduction of blood sugar to levels of 44 to 49 mgm. per cent also provoked changes in the electroencephalogram roughly equivalent to that seen at 16,000 feet.

6. The behavior pattern of the same subjects experiencing equivalent degrees of disturbance in consciousness and equivalent electroencephalographic changes during anoxia, hypoglycemia, and acute alcoholic intoxication was found to vary considerably. This variation was related to the previous personality structure of the subject and the social and psychologic implications of the particular experimental procedures.

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STUDIES IN BLOOD COAGULATION: THE COAGULATION PROPERTIES OF CERTAIN GLOBULIN FRACTIONS OF NORMAL HUMAN PLASMA *IN VITRO*^{1, 2}

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Previous studies from this laboratory have demonstrated that, at least, 2 protein fractions can be prepared from cell and calcium-free human plasma which have a definite effect of lowering the coagulation time of hemophilic blood (1 to 4). One of these protein fractions requires the presence of both calcium ion and prothrombin in order to exhibit its activity. The other acts as a true thrombin since it can convert fibrinogen to fibrin in the absence of calcium and prothrombin.

It has been further demonstrated that the former protein preparation, presumably associated with the euglobulin fraction, can by treatment with chloroform be converted into a protein preparation having proteolytic activity (5, 6). The physiologic rôle of this enzyme has not been determined. However, it is known that it can convert prothrombin to thrombin and hence in appropriate amounts induce blood coagulation (7). In larger amounts it can digest fibrinogen before fibrin formation can take place.

Various preparations of globulin fractions from pooled normal human plasma were placed at our disposal for study of their coagulation properties.³ A summary of our results has been presented by other workers (8).

The present communication concerns the activity of these globulin fractions *in vitro*. In the

accompanying paper a brief preliminary note on the *in vivo* action of one of them is presented.

GENERAL METHODS

All fractions of globulins and parent plasmas were received as dry powder and were put into 4 per cent solution in either isotonic salt solution or in 0.25 per cent sodium citrate in 0.85 per cent sodium chloride solution. The pH of the solutions was adjusted to 7.0 to 7.5 using a glass electrode. The total nitrogen of the final centrifuged solution was determined by a micro Kjeldahl method. The nomenclature used in the description of the fractions was that developed by Cohn and his co-workers (9). Over 140 globulin fractions have been studied. Most of these studies were made during the period of standardization of methods of preparation and will not be reported because the final fractions were not comparable. The fractions presented in this communication are those obtained after the methods of preparation had been satisfactorily standardized so that duplication of results from fraction to fraction was possible.

The average composition of the various fractions mentioned in this paper are stated in Table I.⁴

DEFINITIONS AND METHODS

Fibrinogen is that fraction of the plasma protein which coagulates on the addition of thrombin alone. It was tested for by the addition of 0.1 ml. of a 12.5 per cent solution of a highly potent thrombin solution⁵ to 1 ml. of a 4 per cent solution of the fraction. The time of coagulation, if such occurred, was used as an index of the presence of and a rough quantitative approximation of the amount of fibrinogen.

Prothrombin is the precursor of thrombin. It rapidly develops thrombic activity on the addition of thromboplastin and calcium ions. A similar conversion can be made by the action of certain

¹ This paper is No. 38 in the "Studies of Plasma Proteins" of the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

² The expenses of this investigation were defrayed in part by gifts from the Smith, Kline and French Laboratories, Philadelphia, and in part by a grant "In recognition of Dr. Francis W. Peabody's services to the Foundation" by the Ella Sachs Plotz Foundation.

³ Through the courtesy of Professor E. J. Cohn and Dr. J. T. Edsall.

⁴ These are as given by Cohn, Oncley, Strong, Hughes, and Armstrong (9).

⁵ Lederle "Hemostatic Globulin" (4).

TABLE I
Composition of fractions

Fraction	Albumin	α Globulin	β Globulin	γ Globulin	Fibrinogen
	per cent	per cent	per cent	per cent	per cent
V*	98.5	1.5	0	0	0
IV	15	55	28	2	0
III-2	0	10	75	15	0
III-1	0	4	35	61	0
II	1	0	1	98	0
I	5	4	19	11	61

* Previous studies have shown this fraction devoid of anti-hemophilic properties.

proteolytic enzymes with and without calcium ions (10, 7) and under certain circumstances spontaneously (11). The detection of prothrombin in a fraction of the plasma globulin was made by a modification (12) of Quick's (13) procedure. Instead of the customary 0.1 ml. of plasma, 0.1 ml. of a 4 per cent solution of the fraction was used. When the fraction contained no fibrinogen, this material in the purest form obtainable was added in the reaction vessel before the addition of calcium and thromboplastin. Since all preparations were studied in 4 per cent concentration, the "prothrombin time" was a rough index of the amount present.

Thrombin is that activity developed in plasma which can convert fibrinogen to fibrin without the intervention of either calcium ion or thromboplastin. The presence of thrombin in a fraction was tested for by the addition of 0.5 ml. of a 4 per cent solution of the fraction to 0.5 ml. of a solution of the purest obtainable fibrinogen. The test was always carried out in the presence of an excess of sodium citrate.

Anti-hemophilic activity [plasma thromboplastin of Howell (14)] has been shown to be associated with the plasma euglobulins (1, 3). It is capable in the presence of calcium ions of producing thrombin from prothrombin or alternatively of neutralizing a circulating antithrombin. The anti-hemophilic activity of a fraction was determined by measuring the effect of 0.1 ml. of a 4 per cent solution of the fraction at 37.5° C. and pH 7 to 7.5 on the coagulation time of 2 ml. of hemophilic blood. Usually 2 dilutions of the 4 per cent solution of the protein fraction with 0.85 per cent saline solution to 1/10 and 1/100 of the former concentration were made. When fractions were

extremely active there was marked reduction of the clotting time of the hemophilic blood even at 1/100 dilution. Sometimes activity was found at 1/10 and 1/100 but none when the undiluted material was used. This was presumably due to excessive amounts of citrate ion present in the protein fraction.

Proteolytic activity after chloroform. Twenty-five ml. of a 4 per cent solution of the preparation was shaken briefly with 1/10 its volume of chloroform as previously described (7, 5). When digestion was complete, equal amounts of the chloroform extracts and of 1 per cent casein were mixed and incubated at pH 7 for 96 hours. The increase in non-protein nitrogen was taken as an index of the activity of the proteolytic enzyme content of the plasma. A control consisted of a solution of 1 per cent casein mixed with an equal volume of 0.25 sodium citrate in 0.85 per cent saline. The preparations remained sterile as indicated by bacteriologic examination.

Proteolytic activity without treatment with chloroform. Occasionally certain protein fractions of human plasma have proteolytic activity without previous treatment with chloroform. This activity was tested qualitatively by observing the lytic action of the fraction on a fibrin clot. Quantitatively it was determined by the amount of non-protein nitrogen produced by the addition of a mixture of equal volumes of a 4 per cent solution of the fraction and a 1 per cent casein solution at pH 7.4 at room temperature for 96 hours.

RESULTS

(The complete data are presented in Table II)

Antihemophilic activity. The antihemophilic activity of human plasma was distributed in Fractions I and II + III and was markedly diminished in Fraction IV. From the data of Table II it appears that great activity in reducing the coagulation time of hemophilic blood resides in Fraction I which likewise contains the bulk of the fibrinogen. From a study of runs 119, 123, 124 and 125, it would appear that a marked concentration of the activity over that displayed by the parent plasma was achieved by the method of preparation of Fraction I. However, it is well known (15) that antihemophilic activity rapidly disappears from plasma. Fresh plasma has a

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TABLE II

Coagulation properties of fractions of normal human plasma in vitro

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TABLE II													
Coagulation properties of fractions of normal human plasma in vitro													
Globulin fraction and run	Total protein grams per 100 ml.	pH	Fibrinogen	Proteolysis without CHCl_3	Thrombin	Prothrombin time seconds	Anti-hemophilic			Enzyme activity after CHCl_3 N.P.N.		Remarks	
							Control	Dilution		mgm. produced per 100 ml.	Control		
								0	1-10				1-100
Run 114 II+III IV 1+2+3	2.9 3.6	7.2 7.5	1+ 0	0 —	0 0	19* 30*	105 69	7½ 51	18 58	47 58	31.6 7.4	5.5 0	
Run 116 II+III III 1+2+3 IV-4	2.6 3.1 3.2	7.0 7.0 7.2	1+ ± 0	0 — —	0 0 0	23* 16* 30*	39 40½ 155	6 30 41	16 36 72	22 37½ 110	28.8 32.7 9.9	0 0 0	
Run 117 I II+III IV 1+2+3 IV-4	3.1 2.8 3.2 2.5	7.1 7.0 7.3 7.4	4+ 2+ ± 0	— + 0 —	0 0 0 0	41 22* 28* 49*	40½ 39 155 155	4 7 41 62	7 16 56 85	12 22 110 118	25.6 32.6 24.0 9.8	0 0 0 0	Spontaneous clotting
Run 119 Plasma I II+III IV 1+2+3 IV-4	2.6 2.8 2.8 2.8 1.9	7.3 7.0 7.3 7.3 7.2	3+ 4+ 1+ 0 0	— + 0 — —	0 0 0 0 0	105 68 27* no clot no clot	42 110 55 31 80	49 5 9 31 30	49 14½ 35 34 54	49 14 55 32 72	54.1 13.5 22.9 44.5 9.6	0 0 0 4.1 0.7	Spontaneous clotting
Run 123 Plasma I II+III IV 1+2+3 IV-4	2.6 3.0 2.4 2.9 3.0	7.3 7.5 7.1 7.4 7.1	3+ 4+ 1+ 0 0	— 0 0 0 0	0 0 0 0 0	72 76 37* no clot no clot	42 110 98 31 80	30 6½ 8 31 74	36 9 18 29 75	42 13 53 37 80	61.2 17.5 34.2 52.6 7.0	0 0 0 4.1 0.7	Spontaneous clotting Spontaneous clotting
Run 124 Plasma I II+III IV 1+2+3 IV-4	1.9 2.9 2.8 2.9 2.7	7.0 7.3 7.3 7.2 7.0	3+ 4+ 1+ 0 0	— 0 0 0 0	0 0 0 0 0	96 83 30* 45* no clot	39 100 55 31 80	28 5 14 34 54	23 7 32 29 75	35 13 47 31 80	54.0 8.7 22.1 44.7 20.6	8.4 0 0 4.1 0.7	Fibrinogen = 112.9 mgm. Spontaneous clotting
Run 125 Plasma I II+III IV 1+2+3 IV-4	2.6 3.4 2.4 3.3 2.9	7.2 7.3 7.0 7.1 7.4	3+ 4+ 2+ 0 0	— 0 0 0 0	0 0 0 0 0	29 67 31* 60* 25*	39½ 55 120 110 80	19 6½ 13 33 77	23 8½ 35 57 75	28 12½ 80 110 80	70.6 26.3 18.0 67.2 22.1	8.4 0 0 0 0.7	Fibrinogen = 115.5 mgm. Spontaneous clotting
Run 126 I II-1 II-2 III-1 III-2 IV 1+2+3 IV-4	2.6 2.8 3.3 2.1 2.9 4.0 3.6	7.2 7.1 7.4 7.4 7.0 7.5 7.2	4+ 0 0 0 0 0 0	— 0 — — — — —	0 0 0 0 0 ± 0	120 22* 85* 31* 60* no clot no clot	110 120 98 120 110 110 110	16 120 75 72 8 38½ 82	15 120 85 110 50 79 97	23½ 120 95 120 120 82 106	9.8 0 5.1 19.3 34.1 61.4 59.9	0 0 3.9 2.2 2.2 0 0	
Run 366 II+III II-1 II-2 III-1 III-2	2.6 3.1 3.4 2.3 2.6	7.0 7.0 7.4 7.1 7.4	2+ 0 0 0 0	— — — — +	0 0 0 0 +	25* no clot 88* 25*	65 65 162 162 162	5½ 53 133 50 6	25 53 147 119 30	31 60 147 131 97	23.2 0 0 13.8 38.6	0 0 0 0 0	
* = 0.1 ml. fibrinogen solution added : the usual Quick test.													

* = 0.1 ml. fibrinogen solution added : the usual Quick test.

clotting potency equal to Fraction I. The antihemophilic properties of runs 117, 119, 123, 124, and 125 were in all probability enhanced by the presence of some proteolytic enzyme in the preparation which is known to have some coagulating effect *per se*. While Fraction I is a potent source of antihemophilic material, it should be borne in mind that from 60 to 80 per cent of this fraction is fibrinogen so that the possibility remains that further separation of the components of Fraction I may give rise to greater concentration of this activity.

An analysis of the combined Fraction II and III is shown in runs 126 and 366. Here it is shown that the fraction most active in antihemophilic properties lies in Fraction III, sub-fraction 2. The results are somewhat obscured due to the presence of traces of preformed thrombin in the fraction. Actually the amount present was so small that over 1 hour was required to obtain a clot with pure fibrinogen solution. It is possible, therefore, that while the small amount of thrombin present, and also the small amount of protease, may have enhanced the antihemophilic activity, they did not account for the marked reduction in the clotting time observed. It is also noteworthy that while Fraction III-2 contains antihemophilic activity, it is devoid of fibrinogen.

Plasma proteolytic enzyme. It will be observed that a proteolytic enzyme after treatment with chloroform can be obtained from the parent plasma and from all of the main fractions studied. The parent plasma contains more of the enzyme than any of the sub-fractions. It is to be noted that the protein content of the plasma is in close proximity to the values obtained for the 4 per cent solutions of the fractions. Of the fractions themselves, the greatest concentration of the enzyme is obtained in Fraction IV which is devoid of fibrinogen and of antihemophilic activity. Hence, it would appear that certain fractions can be a good source for the preparation of the plasma proteolytic enzyme in the absence of antihemophilic activity. The amount of prothrombin present is also usually small in Fraction IV. In Fraction III the fibrinolytic enzyme is largely concentrated in Fraction III-2 which also is capable of lytic action on fibrinogen without the previous preparation with chloroform. Similar lytic action is oc-

casionaly found in Fraction I and probably accounts for the instability of solutions of this fraction (16). Spontaneous clotting and lysis of solutions of Fraction I have been frequently observed as indicated in the table.

Prothrombin and thrombin. The plasma prothrombin was found chiefly in Fraction II + III and occasionally in Fraction IV. Only minute traces remained in the parent plasma. Traces of preformed thrombin are occasionally found, chiefly in Fraction III-2.

Fibrinogen. As would be expected from previous reports (9), fibrinogen was largely found in Fraction I with occasional traces present in Fraction II + III. Fraction IV contained no fibrinogen.

DISCUSSION

Interest centers chiefly around the occurrence and distribution of antihemophilic and proteolytic activity in the various fractions and sub-fractions of the plasma globulins. The separation of a highly potent antihemophilic fraction capable of administration in small amounts to hemophilic patients is desirable. Previous investigations at this laboratory have shown that prothrombin and fibrinogen can be separated from the antihemophilic globulin (2). This separation in Fraction I has not yet been accomplished. However, Fraction III-2 contains marked antihemophilic activity but no fibrinogen, so that one may hope for a satisfactory separation in the near future.

The finding that proteolytic activity could be obtained by the treatment of Fraction IV by chloroform is of some academic interest. It clearly shows that fractions containing no antihemophilic activity may be a source of proteolytic enzyme. However, it should be borne in mind that the antihemophilic property of plasma is very labile so that in the preparation of Fraction IV it may have been destroyed. Furthermore, it has been shown (1) that there is a great possibility for adsorption of the antihemophilic property on fibrinogen and since this material has been removed before the preparation of Fraction IV, much of the antihemophilic activity might well be removed with it. The high potency of Fraction I which contains 60 to 70 per cent of the fibrinogen would partially confirm this possibility.

Antihemophilic activity quite often is adsorbed

with prothrombin (1) which may well account for an increase in the activity of Fraction II + III which contains the bulk of the prothrombin. Fraction IV is devoid of both fibrinogen and prothrombin which may well account for the absence of antihemophilic activity in this fraction of the plasma globulin. Another purely speculative reason for the absence of antihemophilic properties in Fraction IV may be that inhibitor materials have been concentrated in this fraction. Such inhibitors are in fact known (16).

The low potency of antihemophilic activity in the parent plasma requires comment. Fresh human plasma is an excellent source of this material. It is known from *in vitro* studies that stored liquid plasma and plasma dried from the frozen state (15) contain minimal amounts of antihemophilic activity. Furthermore, stored whole blood and liquid plasma are not effective *in vivo* in reducing the coagulation time of the blood of hemophilic patients (17). Hence, either in the preparation of Fraction I the plasma contained much more of the antihemophilic material than the specimens examined in the dried state or the concentration of the fibrinogen in this fraction served also to concentrate the minimal amounts of the antihemophilic activity remaining in the parent plasma.

The presence of a protease in Fraction I confirms to a large degree our opinion regarding the cause of the instability of fibrinogen solutions. It has been noted in this laboratory that in the presence of plasma protease fibrinogen solutions clot and the clot lyses within a short time (17).

The foregoing *in vitro* observations suggest that by further subfractionation a highly potent injectable material may be found which may have some value in the treatment of hemophilic patients. Preliminary observations of the effect of injection in hemophilia of a suitable fraction for the commencement of such chemical fractionation are given in the accompanying paper. Furthermore, the data suggest that from Fraction III-2 or Fraction IV a sub-fraction may be obtained which will be a highly potent source of the proteolytic enzyme (7, 5).

CONCLUSIONS

1. The antihemophilic property of normal human plasma appears to be associated with the pro-

teins of Fraction I of Cohn. It is also found in high concentration in Fraction III, sub-fraction 2.

2. The plasma proteolytic enzyme (7, 5) may be prepared from Fractions I, II + III, and IV. In Fractions I and III-2 a proteolytic activity without treatment with chloroform can occasionally be demonstrated.

3. The proteolytic activity after treatment with chloroform can be obtained from Fraction IV which is devoid of antihemophilic activity, fibrinogen and prothrombin.

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THE COAGULATION DEFECT IN HEMOPHILIA: THE EFFECT, IN HEMOPHILIA, OF THE PARENTERAL ADMINISTRATION OF A FRACTION OF THE PLASMA GLOBULINS RICH IN FIBRINOGEN^{1, 2, 3}

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Previous studies from this laboratory have shown that normal human plasma, essentially free from formed elements, fibrinogen and prothrombin, can cause a marked reduction in the coagulation time of the blood in hemophilia when administered intravenously or intramuscularly (1). Furthermore, it was shown that such activity was associated with the plasma euglobulin of normal plasma (2) and was deficient in hemophilic blood (3). The preceding paper (4) has shown that this antihemophilic activity is largely present in Fractions I and III-2, obtained in the separation of the various globulin fractions of the plasma proteins.

The present communication concerns preliminary observations on the effects *in vivo* of the administration in hemophilia of small amounts of Fraction I.⁴ Fraction I contains from 60 to 70 per cent fibrinogen together with smaller amounts of other globulins.

METHODS

Four samples of Fraction I of the plasma globulins, prepared at different times by the Department of Physical

¹ This is paper No. 39 in the "Studies of Plasma Proteins" of the Harvard Medical School, on products developed by the Department of Physical Chemistry, from blood collected by the American Red Cross.

² The products of plasma fractionation employed in this work were developed from blood collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, under contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

³ The expenses of this investigation were defrayed in part by gift from the Smith, Kline and French Laboratories, Philadelphia, and in part by a grant "In recognition of Dr. Francis W. Peabody's services to the Foundation" by the Ella Sachs Plotz Foundation.

⁴ We are indebted to Professor Edwin J. Cohn and Dr. John T. Edsall for furnishing the material on which these observations were made.

Chemistry of the Harvard Medical School (Numbers 162, 163, 171, 172), were used in this investigation. Numbers 162 and 163 were a pool of 7 sub-fractions of Fraction I. Their individual *in vitro* anti-hemophilic activity is shown in Table I. Five patients with classical hemophilia were studied. Sixteen injections of Fraction I were made in varying amounts either intravenously or intramuscularly.

The coagulation time at 37.5° C. of the patient's blood was determined before, and at various intervals after, injection using the method of Pohle and Taylor (5). Prothrombin times were determined at the same time intervals as the coagulation times, using a modification of Quick's method in certain of the studies.

TABLE I

The effect of the addition of 0.1 ml. of the components of run 162 on the coagulation time of 2 ml. of blood (in vitro)

Fraction	Control C.T.*	Antihemophilic activity			T.P.**
		0	1/5 dilution	1/50 dilution	
	minutes	minutes			grams per 100 ml.
162-1	74	53	30	25	1.35
162-2	74	20	22	46	1.29
163-1	74	16½	17½	22	1.04
163-2	74	13½	17	32	1.63
163-3	67	22	19½	19½	1.29
163-4	67	25	19½	24	1.34
163-5	67	26	22	21	1.26

* C.T. = coagulation time.

** T.P. = total protein in original solution of fraction.

The materials used were received as dried powders and dissolved in small amounts of isotonic saline. Preparations of Fractions 162 and 163 were sterilized by passage through a Berkfeld filter. The other fractions were received from the Massachusetts Antitoxin and Vaccine Laboratory as sterile dry powder. These were dissolved in sterile isotonic saline solution and injected without further sterilization. The amounts injected in milligrams of protein are given in Table II.

In 1 patient, a comparison of the coagulation effect of whole blood, plasma and Fraction I was made.

TABLE II

Effect of the parenteral administration of Fraction I on the coagulation time of the blood in hemophilia

Patient	Run no.	Protein injected	Route of injection	Coagulation time											
				Pre-injection (control)	Post-injection										
					10 min.	$\frac{1}{2}$ hr.	1 $\frac{1}{2}$ hr.	4 hrs.	5 hrs.	6 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.	
J. C.	162-163*	11.5	I.V.	96		46		50			112				
J. C.	162-163*	125	I.V.	105	20	23		36			44	72			
W. G.	162-163*	125	I.M.	168	172	140	210								
R. W.	162-163*	125	I.M.	35	10	18	21	40							
J. S.	171	50+150†	I.V.	36		21 $\frac{1}{2}$	18 $\frac{1}{2}$		22 $\frac{1}{2}$	27		38	43		
J. C.	171	50+150†	I.V.	88		21 $\frac{1}{2}$	23		19		21 $\frac{1}{2}$	34	72		
R. W.	171	100	I.V.	33		13 $\frac{1}{2}$				20		25			
R. W.	171	100	I.V.	25		19				25		35			
R. W.	171	100	I.V.	35		19				21 $\frac{1}{2}$		32			
J. C.	171	100	I.V.	82		19				20 $\frac{1}{2}$		38			
J. C.	171	100	I.V.	38		20				37		38 $\frac{1}{2}$			
J. C.	171	100	I.V.	38 $\frac{1}{2}$		17				21		58			
J. S.	171	200	I.V.	43		17				29 $\frac{1}{2}$		35			
T. K.	171	200	I.V.	62		19				21 $\frac{1}{2}$		34	52		
J. C.	172	400	I.V.	100		13 $\frac{1}{2}$				16		34	36	75	
T. K.	172	600	I.V.	52		15				16 $\frac{1}{2}$		44	45		

* Pooled Fraction I. Run 162 (Cut 1 and 2) and Run 163 (Cut 1 to 5).

† 20 minutes between injections.

RESULTS

The data are presented in Table II and Figures 1 and 2. With the exception of patient W. G. in whom the response to the injection was minimal,

the administration of Fraction I produced a prompt and marked fall in the coagulation time of the patient's blood.

In one instance, a dose of 11.5 mgm. of protein

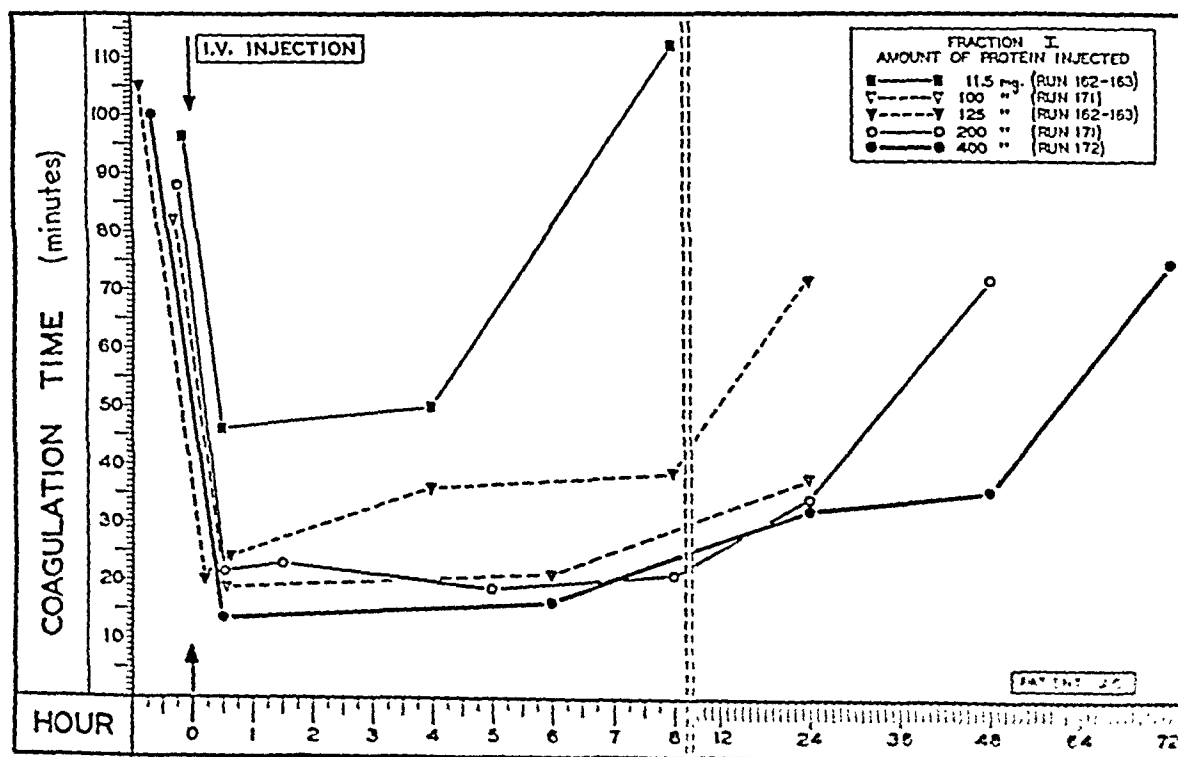


FIG. 1. EFFECT OF PARENTERAL ADMINISTRATION OF FRACTION I ON CLOTTING TIME IN HEMOPHILIA.

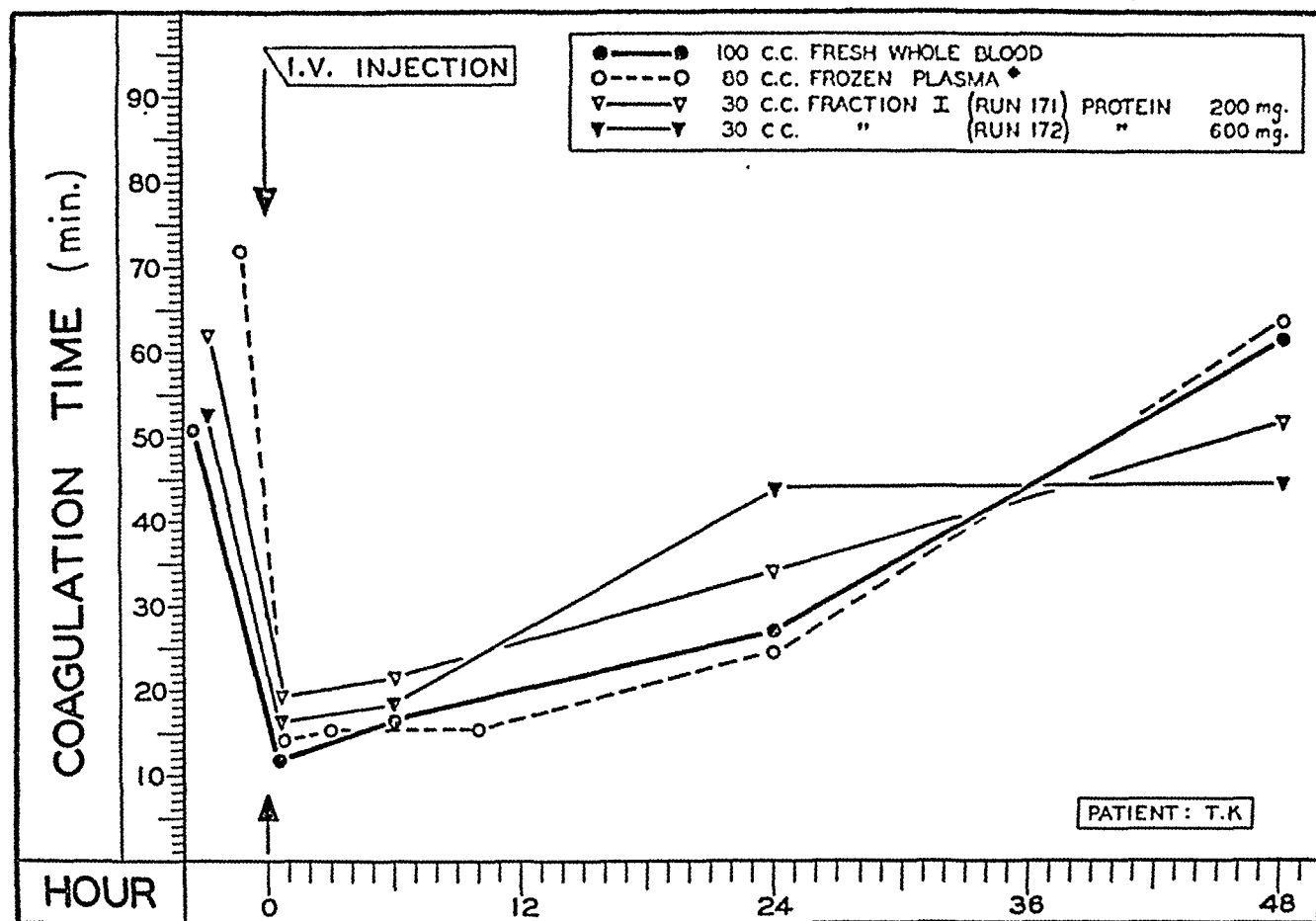


FIG. 2. COMPARISON OF THE EFFECTS OF WHOLE BLOOD, BLOOD PLASMA AND FRACTION I, GIVEN PARENTERALLY, ON CLOTTING TIME IN HEMOPHILIA

* Plasma obtained from fresh whole blood and immediately frozen to minus 20° C.

gave a marked drop in the coagulation time of the patient's blood. The fall was not optimal and the duration of the effect was not long. With amounts between 100 and 200 mgm. of protein the coagulation time fell toward normal limits and occasionally reached them. The duration of the effective reduction for clinical purposes lasted from 6 to 8 hours although the coagulation time of the blood 24 hours after injection was usually prolonged but often shorter than the control coagulation time.

When more than 200 mgm. were injected the blood coagulation time reached normal limits and the evidence indicates a somewhat longer period of effective reduction. The data of Figure 1 indicate the changes in the coagulation time of the blood of patient J. C. using various amounts of Fraction I.

Experience in this laboratory has shown that the injection of 80 ml. of fresh plasma or 100 ml. of whole blood is an effective therapeutic proce-

cedure in reducing the coagulation time of the patient's blood to normal prior to surgical procedure. The effectiveness of 200 and 600 mgm. of the protein of Fraction I were compared to the effectiveness of 80 ml. of plasma and 100 ml. of whole blood. The results are shown in Figure 2. It would appear that from 200 to 600 mgm. of the protein were on the whole just as effective as the amounts of whole blood and plasma generally used in this clinic. The duration of the effect was approximately the same.

As shown by the data of Table I, in 1 patient there was no essential difference between the intramuscular and intravenous routes of administration. There was no change in the prothrombin time of the patients following the administration of the globulin fraction.

It is of some practical interest that the injection of a patient with an active globulin fraction had no influence on the effectiveness of subsequent injections of the material.

DISCUSSION

The preliminary data show that a sharp fall in the coagulation time of the blood in hemophilia follows the intravenous administration of as little as 11.5 mgm. of the protein of Fraction I. Since it is known that 60 to 70 per cent of the Fraction I protein is fibrinogen (6) and that globulin fractions devoid of fibrinogen are active (1), it can be stated reasonably that the amount of anti-hemophilic factor injected could be much smaller than the total amount of protein administered. Hence further separation of the protein of Fraction I may well further reduce the amounts of the active protein required to produce effective reduction of the coagulation time of the blood in hemophilia. Some suggestions that this may eventually be accomplished are given in the preceding paper (4) where it is shown that a fibrinogen-free fraction (Fraction III-2) has marked anti-hemophilic activity *in vitro*.

The hemophilic patient who failed to respond satisfactorily to the injection of 125 mgm. of the protein of Fraction I, responds poorly even to the injection of 250 ml. of normal human plasma. His coagulation time is often 170 minutes. He has not returned to this clinic and thus no information is available as to effect of larger doses. It is possible that even he might have responded to such larger doses of the globulin.

The dosage required of the material is not known and will require a considerable amount of investigation using fractions of the same potency. At present it appears that from 200 to 600 mgm. of the protein are as effective as 80 ml. of plasma or 100 ml. of whole blood.

The only untoward reaction observed was a slight sclerosis of an injected vein in one instance. The degree of sclerosis was of trivial magnitude such as is often encountered following the injection of 50 per cent glucose solution. Since the fraction was administered in high concentration and quite rapidly, future consideration of these points probably will remove this objectionable feature. The same patient has been reinjected on several occasions without this untoward reaction.

These observations clearly indicate that Fraction I of Cohn is an active anti-hemophilic preparation. This communication does not suggest

that it is the most active of the globulin preparations. Our investigations for the future will determine whether other fractions are more or less active than Fraction I.

CONCLUSIONS

The administration, intravenously or intramuscularly, of small amounts of the globulin fraction (Fraction I) of pooled normal human plasma reduced the blood coagulation time of hemophilic blood toward or to normal values in 15 out of 16 instances where it was employed. One of these patients responded to as little as 11.5 mgm. of the globulin. In 1 patient, the effect of the intramuscular administration of 125 mgm. of the protein was minimal. The dosage for therapeutic use has not been established but 200 to 600 mgm. of the globulin have an effect equal to that obtained by 80 ml. of fresh plasma or 100 ml. of whole blood.

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THE INCREASED PLASMA VOLUME IN CARDIAC INSUFFICIENCY: ITS CORRELATION WITH RIGHT-SIDED FAILURE

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It is now well established that in cardiac insufficiency there is an increase in the circulating blood volume as calculated by dye methods (1, 2). The mechanism of the rise in the red cell component has been attributed to anoxemia (3); that of the increase in plasma volume has remained obscure.

Discrepancies in the different quantitative measurements of cardiac insufficiency are not rare. The occasional appearance of acute right-sided failure without a comparable rise in peripheral venous pressure has been observed in this hospital. In addition, others have noted that the plasma volume rise preceded the venous pressure change in several cardiac patients in whom failure was induced by sodium chloride administration (4). Although a general parallelism between the blood volume, circulation time and venous pressure has been reported (1), other workers were unable to determine any significant relationship between these values (2).

Because of the lack of consistent correlation between the various clinical measurements of cardiac insufficiency, the present study was undertaken to determine whether the plasma volume might be related to the degree of circulatory interference in the hepatic and portal vascular beds. This was based on the hypothesis that these areas were larger and more logical sites for the volume increase than the smaller pulmonary and more erratic peripheral circulations.

MATERIAL AND METHODS

Sixteen patients with heart disease were studied on the wards of the Presbyterian Hospital, all having been admitted because of uncomplicated acute cardiac insufficiency and with no evidence of acute or chronic blood loss or shock. Only those patients whose heart failure was predominantly right (portal and systemic congestion) or left-sided (pulmonary congestion) were included, and they were divided into 2 equal groups on this basis. Although several of each group gave a history of previous episodes of cardiac insufficiency, the duration of acute signs or symptoms was not longer than 2 weeks before admission in any instance. Every effort was made

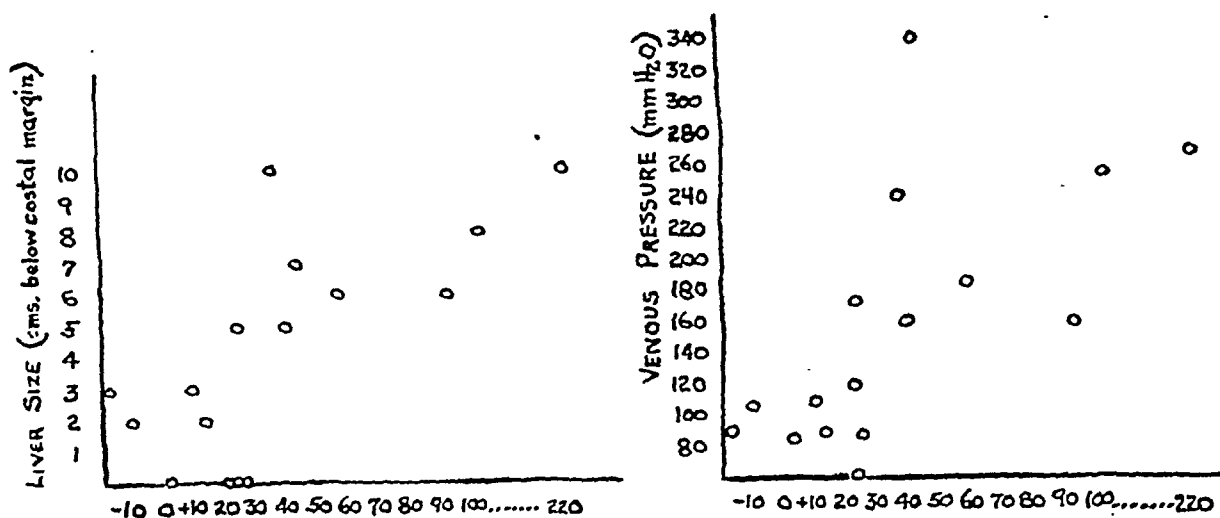
to secure data as soon after hospital admission as possible and before essential therapy had produced any modification of the clinical picture. The average age of the 2 groups of patients was comparable, although they varied in age from the fourth to the seventh decade.

All observations were made within a period of several hours. Blood samples were obtained with the patient lying in bed with a 30° head gatch, and after at least a 20-minute period of inactivity in that position. Circulation times were measured with either sodium dehydrocholate or calcium gluconate. The plasma volume was determined with the blue dye T-1824, the optical density being measured with the photoelectric colorimeter (5). Multiple serum samples were obtained after 10 minutes because of expected variations and delay in mixing. Predictions of plasma volumes were based on the data of Gibson and Evans (6) and the observed hematocrit, using both weight and surface area for comparison. The difference between observed and predicted values was expressed as a percentage deviation from normal. Determinations of red cell volume were not made because of the variable and conflicting results obtained by current methods.

RESULTS

The results are shown in tabular form. It is apparent that the patients with right-sided cardiac insufficiency exhibited a greater increase in plasma volume than did those with left-sided failure. Although the degree of insufficiency was in general reflected in other measurements, such as venous pressure, circulation time, and vital capacity, only a rough parallelism between these values and the plasma volume was observed. Quantitative correlation with liver size was impossible because of difficulties in accurate measurements, because of variations in body build and the position of the diaphragms, and because antecedent liver enlargement could not be excluded. Nevertheless, the rise in plasma volume was also generally related to the degree of liver enlargement (Figure 1).

Predicted plasma volumes based on weight are regarded as unsatisfactory in cardiac patients because of edema. Yet, at times, the comparison between predicted values based on height and surface area showed marked deviation from each



% DEVIATION OF PLASMA VOLUME FROM PREDICTED VALUES (based on height)

FIG. 1. THE RELATIONSHIP OF THE PLASMA VOLUME TO LIVER SIZE AND VENOUS PRESSURE

other in either direction. This suggests that any single method of estimating normal values may sometimes be subject to error and that only major changes in plasma volume can be regarded as significant in a study of this kind.

DISCUSSION

The observation that the increase in plasma volume may be correlated with right-sided cardiac insufficiency is in itself neither surprising nor illogical. Left-sided failure does not permit large volume increases as it requires but small reductions in vital capacity to produce marked symptoms; whereas major increases in congestion in the portal and peripheral bed can be tolerated readily. At autopsy, the liver in cardiac insufficiency may be greatly increased in weight, its vascular channels dilated and congested, and a likely reservoir for many hundred ml. of blood.

The increase in plasma volume noted in this study cannot be explained by loss of dye in edema fluid (1). Although the initial rapid fall in concentration of the dye is delayed in cardiac insufficiency, presumably because of delayed mixing, no positive evidence could be obtained to show that there was any abnormal loss of dye from the circulation. Comparison of the subsequent constant disappearance slope in the patients with increased volumes gave no indication of a faster removal of dye than existed in those with left-sided failure.

As regards the mechanism of the increase in plasma volume in right-sided cardiac insufficiency, it cannot be explained solely by the retention of salt and water. Oliguria or anuria due to other than cardiac failure is not accompanied by changes in plasma volume of the same degree or rapidity as seen in heart failure. It has been suggested that in cardiac insufficiency the kidneys may not be able to excrete salt and water in a normal manner and that this may be a function of decreased cardiac output rather than secondary to congestion (4). The observation that certain patients first gain weight, then increase blood volume, and only later demonstrate a rise in venous pressure, can only be explained by postulating a reduction in renal excretion secondary to heart failure.

There are several reasons which support the hypothesis that the portal and peripheral circulatory beds do not react similarly to the same increased load and that, as reviewed years ago by Krogh, the liver may have potentialities as a variable reservoir (7). These include the absence of any exact linear relationship between the plasma volume and the venous pressure or circulation time, as noted by others (2) and confirmed in this study. When excessive fluids are administered rapidly by vein, using the technique of Caughey (8), it is at times possible in both normal and cardiac patients to see enlargement of

TABLE I									
Clinical and laboratory data in patients with cardiac insufficiency									

GEORGE A. PERERA

TABLE I
Clinical and laboratory data in patients with cardiac insufficiency

Case	Sex	Weight	Height	Surface area	Etiology	Dyspnea	Cyanosis	Rales	Vital capacity	Edema	Liver—below costal margin in mid-clav. line	Ascites	Venous pressure	Circulation time	Hemoglobin	RBC	Serum proteins	Hemato-crit	Plasma volume		Deviation from predicted plasma volume based on	
																			Height	Surface area		
Chiefly left-sided cardiac insufficiency																						
1. C.B.	F	51	161	1.54	hypertension	++	0	++	++	0	0	0	84	9	3.9	6.8	31	2810	2730	2760	+ 3	
2. D.W.	M	79.5	177.5	1.96	hypertension	++	0	++	++	0	0	0	90	13	5.3	6.0	44	3850	3140	3300	+ 17	
3. E.S.	M	70	187	1.96	arteriosclerosis	+++	0	++	++	0	0	0	110	13	4.2	6.1	43	3180	3245	3150	+ 11	
4. L.P.	M	70	177.5	1.86	arteriosclerosis	+++	0	++	++	0	0	0	105	15	5.2	6.4	44	2830	3160	3150	+ 11	
5. A.B.	M	66.3	177.5	1.83	pericarditis	+++	0	++	++	0	0	0	85	15	5.2	6.4	48	3240	3140	3060	+ 8	
6. T.K.	M	69	165	1.76	rheumatic	+++	0	++	++	0	0	0	90	16	5.0	6.0	50	2600	2705	2705	+ 16	
7. J.M.	M	80	174	1.95	arteriosclerosis	+++	0	++	++	0	0	0	105	15	5.2	6.4	44	3180	3160	3150	+ 13	
8. J.T.	M	71	181	1.91	hypertension	+++	0	++	++	0	0	0	90	16	5.0	6.0	50	2865	2865	2865	+ 13	
Chiefly right-sided cardiac insufficiency																						
9. R.V.	M	60	157	1.60	pericarditis	0	0	2500	++	8	0	255	15	15	3.8	4.2	44	4660	2300	2550	+ 83	
10. F.S.	M	61.4	169	1.70	cause?	0	0	1725	++	5	0	175	13.5	13.5	4.5	6.9	38	4050	3255	3075	+ 33	
11. M.R.	F	102.9	155	2.08	arteriosclerosis	0	0	1610	++	10	0	268	14	14	4.8	8.1	48	6760	2110	3240	+ 103	
12. F.C.	M	54	161	1.56	rheumatic	0	0	1625	++	6	0	160	17	17	4.0	6.5	55	2900	2070	3240	+ 40	
13. L.S.	M	80	158	1.82	arteriosclerosis	0	0	2350	++	7	0	340	15	11.6	4.1	7.9	62	3150	1630	1870	+ 55	
14. T.P.	M	74.3	172.5	1.84	interventricular septal defect	0	0	3500	++	10	0	184	10	10	3.8	5.8	42	4450	3145	3135	+ 41	
15. D.D.	F	48	160	1.48	rheumatic	0	0	3200	++	6	0	0	12.3	12.3	4.4	6.2	46	3150	2290	2230	+ 41	
16. J.F.	M	174	174	1.74	rheumatic	0	0	3000	++	6	0	0	12.3	12.3	4.4	6.2	46	4740	2970	2775	+ 71	

the liver without a comparable rise in venous pressure. The clinical observation that pressure on an enlarged liver in cardiac insufficiency produces a rise in venous pressure has been made repeatedly.

It is therefore suggested that the liver and possibly the entire portal circulation react to increases in hepatic vein pressure by dilatation and an enlarged venous bed. Peripheral veins on the other hand, perhaps because of better venous tone or increased pressure from without, respond to the same stimulus primarily by a rise in pressure and with less change in volume. A mechanism such as this may be at least a partial factor to account for the increased plasma volume in cardiac insufficiency.

CONCLUSIONS

1. The increase in plasma volume, frequently encountered in patients with cardiac insufficiency, appears to be associated with right-sided failure.

2. It is suggested that this increase is due to dilatation and engorgement of vascular channels in the liver and portal circulatory beds.

I am indebted to Miss Barbara Wright for assistance in compiling some of the clinical data, and to Dr. Dickinson W. Richards, Jr., for his valuable suggestions.

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THE SOLAR HEAT LOAD: ITS RELATIONSHIP TO TOTAL HEAT LOAD AND ITS RELATIVE IMPORTANCE IN THE DESIGN OF CLOTHING^{1,2}

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(Received for publication March 17, 1945)

This study was undertaken to estimate the relative effect of clothing in protecting men exposed out-of-doors from the heat load contributed by sunlight. Under such conditions, sunlight, both direct and reflected, forms a certain portion of the total heat load. This will be referred to herein as the *solar heat load*. If the influence of clothing on the total heat load is to be analyzed, this factor is best treated as separate from the heat load contributed indirectly by the sun through its influence on the temperature of the ambient air and the terrain.

The evaluation of the effect of clothing on the solar heat load by direct experimental methods presents many difficulties. Sunlight cannot be closely simulated in the laboratory and, on the other hand, testing under outdoor conditions presents difficulties because numerous factors cannot be accurately evaluated and controlled.

THE SOLAR SPECTRUM

In order to view the problem properly, reference must be had to the spectrum of sunlight. Curve 0 in Figure 1 represents the spectral distribution of sunlight outside the earth's atmosphere. The spectral distribution is altered in passage through the atmosphere due to the fact that all wave lengths are not absorbed equally. The atmospheric constituents chiefly responsible for this alteration of the spectrum are ozone, which absorbs the short wave length ultraviolet end of the spectrum, and water vapor which absorbs the long infrared wave lengths. The latter is of particular importance with regard to the present problem. The quantities of both ozone and water vapor in the atmosphere vary at different times

and places, and the spectral distribution of sunlight is altered accordingly. The other gases in the atmosphere absorb very little within the spectral range of sunlight. The spectral distribution is also altered by scattering by gas molecules, and by dust particles. Curves 1 and 2 in Figure 1 represent sunlight at the earth's surface when certain quantities of ozone (2.8 mm.), water vapor (20 mm.), and dust (300 particles per cm³) are present in the atmosphere. Curve 1 represents the spectrum when these atmospheric conditions pertain and when the sun is directly overhead, while curve 2 represents the spectrum under the same conditions when the sun is 60° from zenith, at which time the rays pass through twice as thick a layer of atmosphere.

Considering all these factors, it is obvious that accurate predictions cannot be made about sunlight without direct measurements, or without knowledge of the atmospheric conditions and proper consideration of latitude, season, and time of day, all of which determine the angle of the sun with respect to the zenith.

Figure 1 shows that the maximum of the solar spectrum occurs at about 0.48 μ . Thermal emission having its maximum at this wave length would be given off by a black body at 6,000° K. (K. = Kelvin, absolute temperature). Such a temperature is not attainable in the laboratory for a mass great enough to supply quantities of radiant energy comparable to sunlight. This presents an apparently insurmountable barrier to the simulation of sunlight in the laboratory.

The curves R and C in Figure 1 indicate the spectral sensitivity of, respectively, scotopic vision (rods) and photopic vision (cones). The latter covers the approximate range 0.4 μ to 0.7 μ . This is generally referred to as the visible spectrum, shorter wave lengths being denoted ultraviolet, and longer wave lengths infrared. Measurements in which the human eye is used as the photo-

¹ The opinions or assertions contained herein are the private ones of the writer, and are not to be construed as official or reflecting the views of the Navy Department.

² This paper was originally prepared as a report for the Subcommittee on Clothing of the National Research Council.

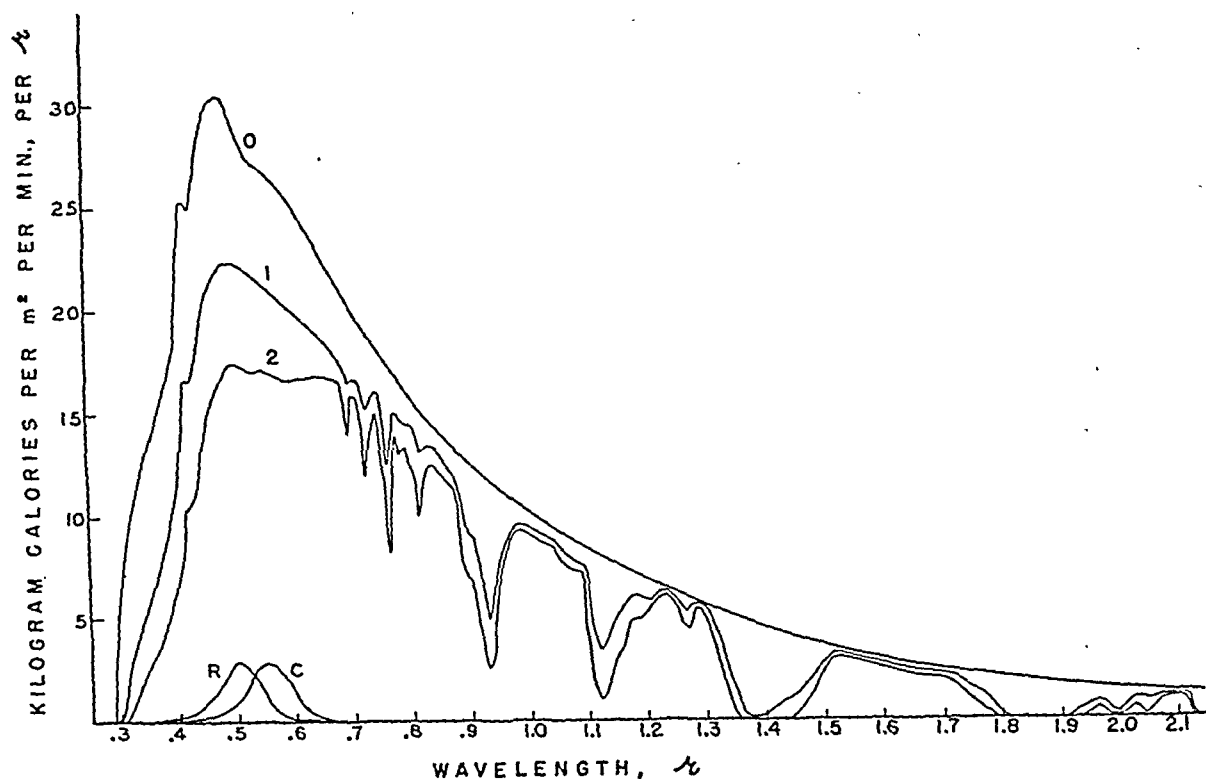


FIG. 1. SPECTRAL DISTRIBUTION OF SUNLIGHT

0, outside the atmosphere; 1, with the sun at zenith; 2, with the sun at 60° from zenith. Curves 1 and 2 are for 20 mm. H_2O , 2.8 mm. ozone, and 300 dust particles per $cm.^2$ From the data of Moon (1).

Curves R and C indicate, respectively, the spectral sensitivity of the human rods, and cones; the ordinate units are arbitrary.

sensitive instrument (this includes all "photometric" measurements) give inaccurate information as to the intensity of the ultraviolet or infrared radiation or of total sunlight.

In the present problem we are concerned with the heat load contributed by sunlight, which is made up of wave lengths ranging from approximately 0.29μ to 2.2μ . A certain portion of the radiation falling upon the body is absorbed, and the remainder reflected. If the body were covered with a surface which reflected a large proportion of all these wave lengths, as for example, with aluminum paint, the solar heat load might be reduced to a negligible quantity. White clothing would reduce the load, but camouflage requirements for military clothing limit the amount of sunlight that can be reflected in certain regions of the spectrum, for a part of the visible spectrum must be absorbed in order that a man may appear to blend into the terrain. Thus, any attempt to

improve the reflecting power of clothing must be limited by the requirements of camouflage so far as visible wave lengths (0.4μ to 0.7μ) are concerned. Since photographic reconnaissance using infrared sensitive photographic emulsions must be considered also, similar restrictions are placed on the amount of reflection allowable in the near infrared, to which these emulsions are sensitive. If the more common infrared sensitive emulsions are used, the long wave length limit is about 0.9μ ; if it is possible to use the most sensitive emulsions, this must be extended to 1.2μ . In Table I the amounts of solar radiant energy falling on a horizontal surface are shown for various spectral regions for different conditions.

REFLECTION OF SUNLIGHT FROM FABRICS

Aldrich has recently measured the reflection of sunlight by a number of fabrics. His measurements, quoted by Wulsin (2), are reproduced in

TABLE I
Energy of sunlight¹

Energy of sunlight				
Zenith angle	All wave lengths	Kilocalories per m. ² per minute		
		Exclusive of visible (all except 0.4 μ to 0.7 μ)	0.7 μ to 0.9 μ	0.9 μ to 1.2 μ
0° ²	14.7	8.7	5.0	2.7
0° ³	13.2	7.3	3.9	1.9
60° ³	10.6	5.9	3.3	1.5

¹ Estimated from the data of Moon (1).

² Dry air, 2.8 mm. ozone, 300 dust particles per cm³.

³ 20 mm. H₂O, 2.8 mm. ozone, 300 dust particles per cm³.

Table II, together with a few older measurements (3). Earlier measurements by Coblenz (4) are in general agreement with these, but refer to only limited portions of the spectrum. Aldrich's data include measurements of transmission of sunlight by the fabrics, which in no instance is high. It may be assumed that most of the transmitted radiation is eventually absorbed either at the skin surface or by the fabric, so it has been included in the percentage contributing to the heat load in the first column of Table II.

TABLE II
Reflection of total sunlight by various fabrics

Item	Fabric	Contributing to the heat load ¹	Reflected	Transmitted
			per cent	
1	<i>Data of Aldrich</i> Shirt, Mock Leno, slightly permeable	55.9	44.1	5.1
2	Cotton, khaki—8.2 oz.	43.7	56.3	0.0
3	Cotton, percale, white	33.2	66.8	0.5
4	Cotton, percale, O.D.	51.5	48.5	2.5
5	Cotton, tubular balbriggan	37.6	62.4	3.2
6	Cotton, twill, khaki	48.3	51.7	0.2
7	Cotton, shirting worsted, O.D.	61.1	38.9	0.1
8	Cotton denim, blue	67.4	32.6	0.0
9	Cotton, herringbone twill	73.7	26.3	0.1
10	Cotton, duck No. 746	92.8	7.2	0.0
11	<i>Data of Martin (3)</i> Cotton shirt, white unstarched, 2 thicknesses	29.0	71.0	
12	Cotton shirt, khaki	57.0	43.0	
13	Flannel suiting, dark gray	88.0	12.0	
14	Dress suit	95.0	5.0	

¹ The transmitted radiation is considered to be absorbed by the skin (see text).

There is considerable difference in reflection by the different fabrics. As would be expected, white fabrics reflect more than colored fabrics, but the total reflection need not parallel too closely the apparent darkness to the eye. Aldrich has estimated the per cent of radiation reflected in the "visible" (0.3 μ to 0.7 μ) and in the "infrared" (0.7 μ to 2.5 μ), for the items described in Table II, and these data are reproduced in Table III. In general, these fabrics reflect infrared radiation to a greater extent than visible. This is contrary to a widespread, erroneous belief that all substances absorb infrared radiation almost completely.

TABLE III
Reflection of visible and infrared portions of sunlight by fabrics

Item	Data of Aldrich Reflection of sunlight "Visible" 0.3 μ to 0.7 μ	"Infrared" 0.7 μ to 2.5 μ
	per cent	per cent
1	24.1	53.7
2	27.8	64.5
3	69.3	60.2
4	28.8	55.0
5	62.7	58.3
6	25.8	58.9
7	72.1	49.0
8	12.1	49.0
9	13.3	30.2
10	6.6	7.5

Improvement of the reflecting power of clothing within the limits imposed by military field requirements would depend chiefly upon finding dyes which, while presenting appropriate colors to the eye (or contrasts to the photographic emulsion) compatible with camouflage requirements, permit greatest reflection of the total radiation. This would entail mainly the reflection of infrared radiation. Texture of the fabrics is also of importance, since some will be better diffuse reflectors than others. These will probably be minor factors, however, and the absorption spectra of the dyes can be regarded as placing the limits of attainable reflection. The absorption spectra of dyes, and hence their reflecting properties, depend upon their chemical constitution. As a rule they do not give sharp spectral cut-offs. It would probably be difficult to predict the appropriateness of particular dyes without laborious study of their absorption spectra, including ranges outside the scope of the usual spectrographic equipment.

Thus, the selection of dyes to improve the reflecting powers of military fabrics would be a difficult task, and the degree of success to be expected is not great.

Under the field conditions, clothing becomes soiled and this may alter both the total reflection and the reflection in different spectral regions.

REFLECTION BY HUMAN SKIN

The reflection of sunlight by human skin provides a basis of comparison with the reflection by fabrics. Martin (3) found 43 per cent reflection of total sunlight from average blond human skin. Brunet skin showed 35 per cent reflection, and negro skin 16 per cent. The values for white skin are in general agreement with those of another investigator (5) for reflection of total sunlight, and compatible with those of others who have measured the reflection of visible, ultraviolet, and infrared wave lengths (6, 7, 8).

THE SOLAR HEAT LOAD AND ITS RELATIVE IMPORTANCE

The total solar heat load, L , impinging upon a man exposed directly to the sun may be divided into 3 portions: D , the direct radiation which strikes the profile exposed; H , the reflected radiation from the sky; and T , the radiation reflected from the terrain. Thus,

$$L = D + H + T \quad (1)$$

While a great many data have been collected on the direct and "sky" radiation falling upon a horizontal surface, there is little information available as regards the sunlight reflected from the earth, or the total energy from these 3 sources which falls upon a solid object, such as the human body. The relative importance of the 3 factors, direct, sky, and earth radiation, varies with the position of the man exposed to them. Hence, integrated measurements of the energy from the 3 sources by means of a physical instrument, such as the Vernon sphere, are not directly interpretable in terms of the solar heat load received by a man exposed to the same conditions. The following estimates in which the human body is treated as though made up of simple geometrical surfaces give an idea of the variations of the solar heat load with various conditions, and provide approxi-

mate values for comparison with the metabolic heat load.

The direct radiation. Let us designate as S , the total energy of all wave lengths contained in sunlight (approximately 0.29μ to 2.2μ) falling on unit area of a surface normal to the sun's rays in unit time. Let F represent the fraction of sunlight diffusely reflected by a fabric or by human skin; the portion of the incident energy absorbed by the clothing or body is then $(1-F)$.³

The direct component of the solar heat load, D , is then

$$D = S(1-F)P \quad (2)$$

where P is the profile exposed, *i.e.*, the projection of the body shadow on a plane normal to the sun's rays. With the sun directly overhead and the man standing erect, P is equal to about 7 per cent of the body surface or about 0.12 m^2 for a man of average body surface, 1.7 m^2 . For a man lying prone, P is equal to about 30 per cent of the body surface or for an average man, 0.51 m^2 . As the sun moves away from the zenith, P approaches 0.51 m^2 for a man facing the sun, approximately as the sine of the zenith angle. At 15° from zenith (one hour) the profile presented should be about 0.13 m^2 , *i.e.*, about the same as when the sun is at zenith. At 60° from zenith (four hours), however, the profile should be about $0.51 \times \sin 60^\circ = 0.42 \text{ m}^2$.

For a man lying prone P decreases as the cosine of the zenith angle, so that when the sun is at 60° the profile presented is only one-half as great as when the sun is at zenith, *i.e.*, 0.255 m^2 . Direct solar heat loads have been calculated on the above basis for 0° and 60° zenith angle, and these appear in Table IV. In all calculations in Table IV the values of solar radiation for 20 mm. water vapor, which appear in Table I have been used;

$$S = \int_{\lambda_1}^{\lambda_2} S_\lambda d\lambda, \text{ and } F = \frac{\int_{\lambda_1}^{\lambda_2} F_\lambda S_\lambda d\lambda}{S}$$

when S_λ and F_λ are, respectively, the solar energy and the reflection for wavelength λ . Since S_λ and F_λ vary independently with λ the numerical value of D in equation (1) depends upon a given set of conditions throughout which the sunlight spectrum varies and the reflection spectrum is different for each fabric; such estimates are only approximate; but the error is certainly not greater than errors introduced by other assumptions that must be made in such an analysis.

and the reflection factor F taken as 0.43, the value found by Martin for blond human skin.

Rough as these estimates are, they show clearly that the direct solar heat load must vary considerably with the position of the man and the time of day; and that the maximum direct load may be received in one position at one time of day, and in another position at another time.

The "sky" radiation. Direct measurements show that about 15 per cent of the radiation falling on a horizontal surface when the zenith angle is between 0° and 60° is reflected radiation from the sky (see 9, p. 60). The proportion of sky radiation increases rapidly for zenith angles greater than 60° , but between 0° and 60° the sky radiation falling on a horizontal surface of unit area should be equal to approximately $\left(\frac{0.15}{1.00 - 0.15}\right) S \cos z$, where z is the zenith angle. The sky radiation falling on a vertical surface will be only one-half that striking a horizontal surface, since the former presents itself to only one-half the heavens. This makes it difficult to estimate the amount of sky radiation striking an irregular body such as a man. In calculating the heat load we have used one-half the total body surface for both the erect and prone positions, on the assumption that about half the body is presented horizontally to the sky when prone and that the greater part of the body surface is presented vertically to the sky when erect. Estimates based on these assumptions are presented in Table IV.

TABLE IV
Estimated solar heat load under various conditions

Position of man	Zenith angle	Solar heat load ¹			
		Direct (D)	Sky (H)	Terrain ² (T)	Total (L)
Erect	0°	0.90	1.13	1.88	3.91
	60°	2.67	0.45	0.75	3.87
Prone	0°	3.84	1.13	0.78	5.75
	60°	1.54	0.45	0.31	2.30

¹ Under the following atmospheric conditions, 20 mm. H_2O , 2.8 mm. ozone, 300 dust particles per cm^3 , and assuming that 43 per cent of the total solar radiation is reflected by the body.

² Albedo of terrain assumed to be 0.25.

The terrain reflection. Estimation of the heat load reflected from the terrain is yet another prob-

lem. It is necessary, first, to know the albedo, A , or fraction of the solar radiation that is diffusely reflected by the terrain.⁴ A good many determinations of the albedos of terrains have been made by visual photometry, and hence can only be accepted as approximate values for total sunlight. For our estimates in Table IV, 25 per cent diffuse reflection has been assumed. This value was obtained for a desert sand by Mr. Irving F. Hand (personal communication). Hulburt (10) obtained somewhat higher values for beach sands. Coblenz found 30 per cent diffuse reflection from the leaves of the tulip tree, but lower values for other foliage. Some high albedos have been obtained for snow, in the ultraviolet and visible, but the infrared is largely absorbed (see 10).

For approximate estimates it may be assumed that the terrain is a surface of infinite area, which reflects 25 per cent of the solar radiation falling on it, including direct and "sky" radiation. A horizontal plane facing this surface will receive per unit area that quantity of radiant energy reflected from a similar area of the reflecting surface; whereas a horizontal plane facing upwards will receive none of the reflected radiation. A vertical plane will receive one-half the radiation received by a horizontal plane facing the reflecting terrain.

If we assume that in the erect position most of the body surface is exposed vertically, we may write

$$T = M(1 - F) \times \frac{A \left(S \cos z + \frac{0.15}{1.00 - 0.15} S \cos z \right)}{2} \quad (3)$$

where M is the portion of the body surface exposed to the diffusely reflected radiation from the terrain. Assuming that all the surface is exposed vertically the value $1.7 m^2$ may be assigned to M . Since any part of the body exposed horizontally facing the earth's surface will receive twice this much reflected radiation from the terrain whereas those that face upward will receive none at all, this assumption seems not too unreasonable.

$$A = \frac{\int_{\lambda_{.29\mu}}^{\lambda_{2.2\mu}} A_{\lambda} S_{\lambda} d\lambda}{S}$$

Hence the same qualifications apply as for S and F , see footnote 3.

In the prone position, the surface presented to the terrain is relatively small. Assuming that a profile 0.5 m^2 is in contact with the ground and another equal profile is presented to the sky, 1.0 m^2 of the body surface will receive no appreciable amount of reflected radiation from the terrain. The remainder of the body surface, 0.7 m^2 may be regarded as presenting a vertical surface, and hence may be substituted for M in equation (3).

In Table IV estimates of the direct, sky, terrain, and total heat loads for the erect and prone positions and for 0° and 60° zenith angle, are presented. Reference to this table indicates that, even though considerable errors may have been introduced in estimating the heat loads from the sky and from the terrain, these factors cannot be neglected in the estimation of the total solar heat load. They also show that these factors may be expected to have very different relative importance under different conditions. This alone throws doubt on the possibility of obtaining satisfactory estimates of the solar heat load by means of experiments in which men are exposed to sunlight out of doors.

THE RELATIVE IMPORTANCE OF THE SOLAR HEAT LOAD

The relative importance of the solar heat load may best be evaluated by comparing it with the heat load of human metabolism. The metabolism of a man of average height and weight is about 96 kilocalories per hour when seated and about 265 kilocalories per hour when marching at 3 miles per hour. For comparative purposes the average of all the values for the total solar heat load presented in Table IV may be used. This is roughly 4 kilocalories per minute or 240 kilocalories per hour. This is 2 to 3 times the resting metabolism, and about equal to the marching metabolism. It would be necessary to evaporate approximately 420 grams of water per hour to take care of the solar heat load of 240 kilocalories. This is about one-half the water requirement of a man marching in the desert in the middle of the day under average summer conditions (5, 11).

To what extent may this heat load be decreased by choosing clothing with the best reflection characteristics? The values for the heat load calculated in Table IV are based on reflection of 43 per

cent. If the reflection were 71 per cent, as measured by Martin for white cloth, the solar heat load would be about one-half or 120 kilocalories per hour. This would seem to be about the best achievable condition, but would not be compatible with military field requirements. On the other hand, if the reflection were 12 per cent, as found for dark flannel suiting, the solar heat load would be increased to about 370 kilocalories per hour. In terms of evaporation of water, this means a difference of about 420 grams per hour as the range between the best and the worst conditions. As regards military fabrics the limits entailed by the requirements of camouflage, and the nature of fabrics and dyestuffs, the differences between field uniform fabrics in terms of the saving of water by reflection of the solar heat load would probably be much less than this.⁵ Reference to Table I will show that if because of camouflage requirements, the saving must be made principally from the longer wave length infrared, it could not be very great in any case.

EXPERIMENTAL DETERMINATION OF THE EFFECTS OF CLOTHING ON THE SOLAR HEAT LOAD

Physiological measurements. It is generally assumed that when the air is relatively dry and the ambient temperature is near that of the body's surface, the amount of water evaporated, as measured by the decrease of body weight, provides a measure of the amount of heat which the body has dissipated, within a given time. This is true only when surrounding surfaces and objects are also at the temperature of the body's surface. It does not imply that it is possible accurately to estimate the solar heat load by comparing evaporative losses for men in the sun and in the shade as has been attempted. In the first place, the establishment of adequate shade for such an experiment is difficult, since reflection of sunlight from the sky and from the terrain which remain when the direct sunlight is eliminated are difficult to evaluate. Furthermore, the use of any object for shading the body introduces another factor, the

⁵ The solar heat load may be easily estimated for the various types of clothing described in Table II. The values of L presented in Table IV need only be multiplied by percentage reflection.

radiation reemitted by that object, and there are still other factors which need to be taken into consideration.

Under conditions in which the ambient air temperature is below body temperature, heat is lost by convection and conduction, which thus interfere with estimates of the solar heat load. Convection, provided by wind or simply by body movement, may be a factor even when the ambient temperature is above that of the body since it may affect the rate of evaporation on the body surface in the case of porous clothing. The estimation of these factors is beyond the scope of this paper, but they should be considered in any calculation of the total heat load.

Another factor seldom taken into account is the exchange of radiation of longer wave lengths than those found in sunlight, between the body and its surroundings, *i.e.*, the terrain and the atmosphere. To appreciate this phase of the problem let us first consider the exchange between the body and the terrain. For the purpose, the terrain may be assumed to be a diffusely radiating surface of infinite extent, in which case the same geometry applies as for the reflection of sunlight from the terrain (see p. 716). On the basis of the assumptions made above, a man standing erect would present his body surface vertically and would receive one-half the radiation from the terrain. We may thus treat the problem as the exchange of radiation between two surfaces of area equal to one-half the body surface or 0.85 m^2 . If the air were absolutely dry, these two surfaces might be treated as black body radiators, and the Stefan-Boltzmann law applied. This law states that the exchange of radiation between two such bodies is proportional to the fourth power of the difference between their absolute temperatures. The magnitude and direction of this heat exchange would depend, upon the temperature of the body surface and that of the terrain. By way of example, if the body surface were at 37° C . and the terrain at 60° C . the body of a man standing erect should gain 128 calories per hour from the terrain, a sizeable addition to the total heat load. If the terrain were cooler than the body, the latter would lose heat.

When water vapor is present a certain fraction of this radiation will be absorbed by the atmos-

phere lying between the body and the terrain. Black bodies at the temperatures of the human body and the terrain, emit radiation over a broad range with a maximum at about 10μ .⁶ Water vapor is transparent to a wide spectral band at about this wave length, but strongly absorbs wave lengths on both sides including a large fraction of that radiated by such bodies (12, 13). The other gases of the atmosphere do not absorb in the spectral region to which water vapor is transparent, with the exception of ozone which is only present in important concentration in the upper layers of the atmosphere. Because of this specific absorption of certain wave lengths the Stefan-Boltzmann law is not directly applicable when water vapor is present in the atmosphere, and the estimation of the heat load emitted by the terrain thus involves considerable uncertainty under these conditions. However, since most of the radiation from the terrain which strikes the body comes from the relatively near regions, the effect of absorption by water vapor may not be great.

TABLE V
Reflection by military fabrics of radiation from a black body at 60° C .

Data of Aldrich			
Item	Contributing to the heat load ¹	Transmitted	Reflected
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	87.0	4.6	13.0
2	90.0	0.6	10.0
3	74.8	0.6	25.2
4	75.0	2.4	25.0
5	90.5	1.5	9.5
6	88.5	0.0	11.5
7	90.4	0.0	9.6
8	90.0	0.0	10.0
9	81.0	0.0	19.0
10	90.0	0.0	9.1

¹ The transmitted radiation is considered to be absorbed since it will be largely absorbed by the skin.

It is improbable that the heat load received by such radiation from the terrain can be reduced appreciably by choice of fabrics. Aldrich has made measurements of the reflection by those military fabrics listed in Tables II and III of radiation from a body at 60° C .; these are presented in Table V. Very little of such radiation is reflected by any of the fabrics.

⁶ The distribution is described by Planck's equation.

Radiation exchange exclusive of sunlight, between the body and the atmosphere involves the same factors, but is even more complex. The transparency of water vapor permits some of the radiation from the body to pass to higher layers of the atmosphere which are cooler than the ambient layer. This is a channel of heat loss usually disregarded. Accurate estimate of this radiant energy loss is difficult, but an idea of its relative magnitude may be gained from an analysis made by Simpson (14, 15) for an entirely different purpose. In considering the heat loss from the earth, this investigator (15) estimates maximum and minimum values for atmospheric transmission taking differences in amount of water vapor into account, and arrives at mean and limiting values for the long wave-length radiant energy lost to the heavens by a horizontal surface at a given temperature (Figure 2). This is generally known as

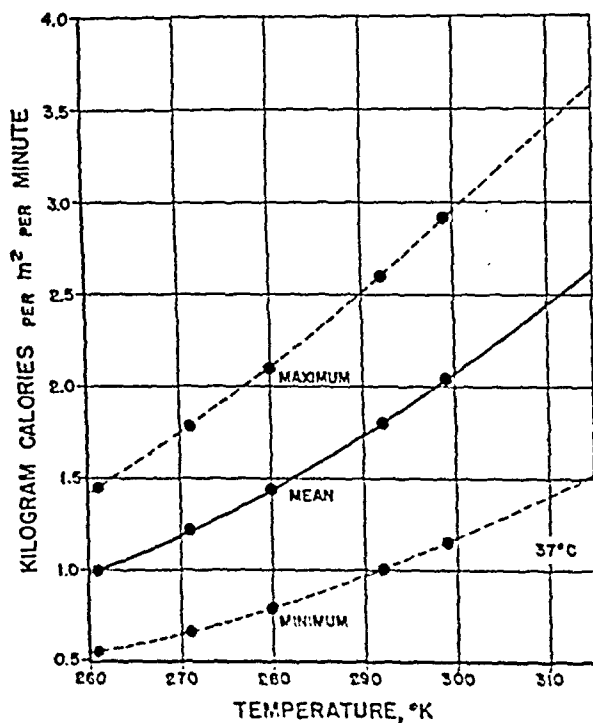


FIG. 2. ESTIMATED RADIATION LOSS FROM A HORIZONTAL SURFACE TO THE ATMOSPHERE

From the data of Simpson (15).

the "nocturnal" radiation because it is usually measured at night. The out-going radiation measured independently of solar radiation during the day is comparable, and is dependent chiefly on the

temperature and humidity (16). Measurements by different methods (17, 18) give values falling within Simpson's estimates.

Extrapolating Simpson's mean curve (Figure 2) we see that a surface at 37° C. may be expected to lose about 2.5 kilocalories per m.² per minute by this channel. Using the same treatment as for solar radiation reflected from the sky a man standing erect would present 1.7 m.² vertically to the heavens and should lose about 128 kilocalories per hour as long wave length radiation. This might be considerably higher or lower depending on the amount of water vapor in the atmosphere.

For purely illustrative purposes, a thermodynamic balance sheet has been attempted in Table VI, for a hypothetical set of conditions, namely;

TABLE VI

Attempted thermodynamic balance sheet for a man marching at 3 miles per hour; ambient air dry with temperature about 37° C., terrain at 60° C., and body surface at 37° C. Sun at zenith

	kilocalories per hour
Metabolism	+265
Total solar heat load	+234
Long wavelength radiation exchange with terrain	+128
Long wavelength radiation exchange with heavens	-128
Evaporation	-506 ¹
Convection and conduction	±?
Total	-7±? (this close apparent balance is fortuitous)

¹ Based on average value from Adolph *et al.* (11), 882 grams of water loss per hour.

sun at zenith, temperature of the terrain 60° C., ambient air relatively dry and at a temperature somewhat above that of the body, the man erect, marching at 3 miles per hour. The evaporation factor is based on the loss of 882 grams of water per hour, an average figure obtained (11) for men walking on the desert. Convection and conduction losses are assumed to be small because the temperature of the ambient air is near that of the body, but represent an unknown value. The radiation values are those calculated in this paper.

The close over-all balance obtained is fortuitous, as is the exact balance between radiation from the terrain and to the heavens. Had the ground temperature been taken as 10° lower or the assumption made that the sun had warmed the clothing

to a temperature 10° higher than that chosen, the balance would be considerably upset. It should be pointed out that for a man at rest, the long wave length radiation exchange would be more important relative to the metabolism, and it might be interesting to explore other possibilities. However, Table VI shows clearly that a balance is possible with values of these magnitudes, but that none of the various items estimated therein can be neglected in drawing up a balance sheet.

The evaporation factor tends to adjust itself due to sweating so that the body temperature does not rise excessively. Thus this factor may be expected to vary to compensate when the other factors shift with various conditions. When the magnitude and variability of the other factors are considered, it does not seem surprising that Adolph and his coworkers (5, 11), should have obtained different values for evaporative heat loss under the various conditions they explored, nor that these values display the general consistency they do.

The whole problem of radiant exchange with outdoor surroundings is, thus, quite complex and cannot be accurately simulated in an enclosed room. Moreover, all these factors render physiological measurements out-of-doors subject to considerable variability, not only in so far as the solar heat load is concerned, but with regard to the heat load as a whole.

Physical measurements. Physical measurements would seem better than physiological for determining protection afforded by clothing against solar heat load, since the important factor to be measured is the diffuse reflection of solar radiation. Such measurements must either be made with sunlight, as were those of Aldrich and Martin, or must involve measurements throughout the spectrum. If sunlight is used, conditions should be selected such that its intensity and spectrum can be estimated with reasonable accuracy. The data of Moon (1) should be useful in this respect. Spectral measurements must cover the range 0.029μ to 2.2μ . All such measurements should be based on the response of thermocouple or bolometer. Determinations made by photometric methods would have little meaning, since they only give an index of the reflection in the visible in terms of the human eye. Since the

fabrics may transmit a certain small fraction of the incident light, which will in turn be absorbed or reflected by the underclothing or skin, the fabric to be tested should be backed with underclothing and/or a surface resembling human skin.

CONCLUSIONS

Since the amount of saving of solar heat load to be anticipated by improvement of the reflecting properties of military uniform fabrics is not great, it would seem wise to concentrate effort on the evaluation of properties of fabrics that can be studied in the laboratory, and which are of importance under all conditions of hot environment, namely, their effect on cooling by conduction, convection, and radiation at ordinary temperatures.

Where reflecting properties of clothing are to be considered, they should be determined by direct physical measurement.

Necessary data are lacking for evaluation of the thermal relationships of man with an outdoor environment, some of which lie in a domain that is generally left to the physicist, the meteorologist, or the astronomer. It would seem important to obtain some of these data with the express problems of the environmental physiologist and climatologist in mind.

SUMMARY

1. The solar heat load received by man is estimated and compared with the metabolic heat load.

2. Methods of determining the effect of clothing on the solar heat load are considered.

- a. Physiological determinations either indoors or out-of-doors are not trustworthy. It is impossible to simulate accurately in the laboratory, either sunlight or the conditions of exposure to sunlight. The variables entering into outdoor experiments render them susceptible to misinterpretation.

- b. Physical measurements of the reflection of sunlight by fabrics should give the best estimates of their effectiveness in combatting solar heat load.

3. The saving of solar heat load to be accomplished under the limitations of camouflage requirements for military clothing may not justify the time and effort needed to make extensive studies.

Most of the values for reflection of sunlight by fabrics used in this report were obtained by Dr. L. B. Aldrich of the Smithsonian Institution of Washington at the request of Dr. F. B. Wulsin of the Military Planning Division, Office of the Quartermaster General. Albedo measurements of terrain made by Mr. Irving F. Hand of the U. S. Weather Bureau have also been employed. It is a pleasure to acknowledge the cooperation of these men in placing their material at my disposal. Thanks are also due to Professors L. H. Newburgh and C. P. Yaglou, and to Commander R. H. Lee, H-V(S), USNR, for advice and criticism.

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SERUM IODINE OF EUTHYROID SUBJECTS TREATED WITH DESICCATED THYROID^{1,2}

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The changes in serum iodine and basal metabolic rate which occur when myxedematous patients are treated with desiccated thyroid have been reported in the preceding paper (1). Many euthyroid subjects differ from myxedematous patients in their ability to tolerate comparatively large amounts of dried thyroid without manifesting significant signs or symptoms of hypermetabolism (2). In the present paper, the influence of thyroid feeding on serum iodine as well as on basal metabolism of euthyroid subjects is described, and the mechanism of euthyroid tolerance to large doses of desiccated thyroid is discussed.

MATERIALS AND METHODS

Data from 2 groups of patients are presented.

The first group consisted of 4 female schizophrenic patients in the Fairfield State Hospital, who were chosen for their willingness to cooperate in an extended experimental study. None of the 4 showed any evidence of thyroid dysfunction, and all were in reasonably good physical condition. The medical and psychiatric status of each patient is summarized at the end of the paper.

The basal metabolic rate of each patient was determined once a week. Duplicate 8-minute tests were performed using the Sanborn Motor-Grafic metabolism apparatus. Patients were required to rest in bed for 1½ hours before the test. The lower of the 2 rates determined during each test period was used rather than the average of both readings. Basal metabolic rates of patients Pa., Ve., and Vi. were usually quite satisfactorily measured, those of Wi. less accurately. Body weight, pulse rate, oral temperature, and blood pressure were measured under basal conditions. Concentrations of precipitable iodine and of filtrable iodine in serum from venous blood were determined each week by methods previously described (3, 4). If the serum could not be precipitated at once it was placed in a securely-stoppered

50 ml. Erlenmeyer flask and stored in a quick-freezing chamber. When frozen sera were analyzed, great care was exercised to agitate the thawed serum thoroughly before taking duplicates for analysis, because during freezing the serum proteins are concentrated at the bottom of the flask. The accuracy of serum iodine determination was about ± 0.5 gamma per cent. Serum total proteins were determined each week by the falling drop method of Barbour and Hamilton (5). Serum total cholesterol and serum titrated fatty acids were determined at monthly intervals by methods previously described (6, 7). Complete blood counts were done every 2 or 3 weeks throughout the course of the investigation. The first 5 weeks of the study of each patient constituted a control period. U.S.P. desiccated thyroid* was then given daily in powdered form as a suspension in a small amount of water under the close supervision of a graduate nurse or of an experienced head attendant. The dose was increased stepwise at 4-week intervals from 3 to 6, 10, 15, and finally to 20 or 25 grains per day. The highest dose was maintained for 6 to 8 weeks, and then abruptly discontinued. Thereafter the patients were followed until no further significant changes in serum iodine or basal metabolic rate were observed. Minor individual variations from the program outlined above appear in Figure 1.

The second group consisted of 9 ambulatory patients followed for considerable periods of time in the metabolism clinic of the New Haven Hospital. The clinical status of each of these patients is described in the legend of Figure 2. Determinations of basal metabolic rate and serum total iodine were made at approximately monthly intervals. Barring unusual iodine intake, serum total iodine does not differ significantly from serum precipitable iodine. The dose of thyroid and the duration of treatment of each patient are indicated in Figure 2. Data concerning the metabolic response of 3 of these subjects to various doses of thyroid have already been published (2).

RESULTS

A. Fairfield State Hospital Patients

The changes in basal metabolic rate, serum precipitable iodine and other significant variables before, during, and after administration of desiccated thyroid to the 4 schizophrenic patients are depicted in Figure 1. In general the signs and

¹ This investigation was aided by a grant from the Fluid Research Funds of the Yale University School of Medicine.

² A preliminary report of this work has appeared in the Proceedings of the American Society for Clinical Investigation in the Journal of Clinical Investigation, 1944, 23, 931.

* Parke-Davis Brand.

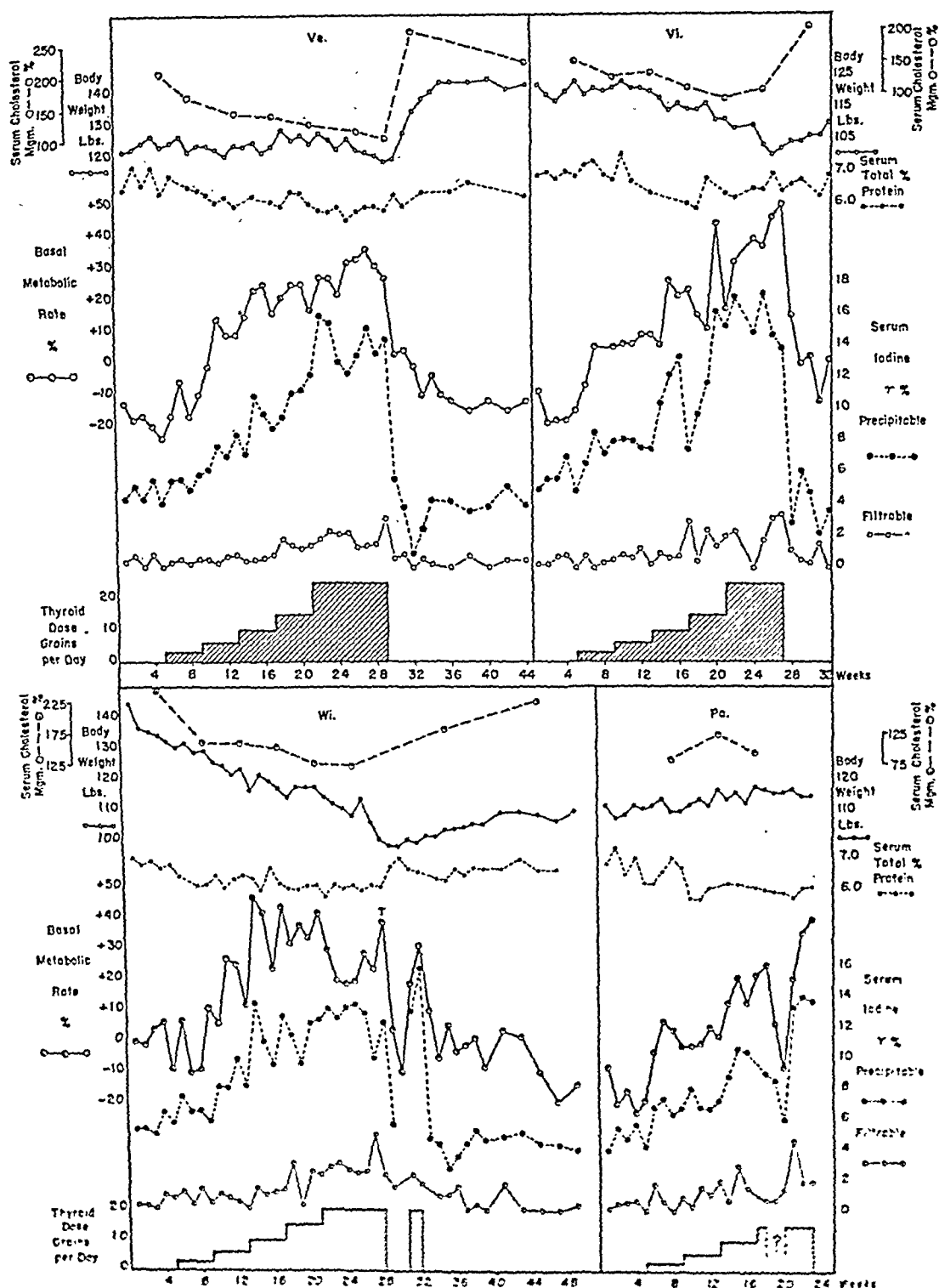


FIG. 1. THE EFFECT OF LARGE DOSES OF DESICCATED THYROID ON SERUM IODINE, BASAL METABOLIC RATE, AND OTHER VARIABLES IN THE EUTHYROID SCHIZOPHRENIC PATIENTS.

The significant changes depicted in these graphs are discussed in the text. Each point represents a different determination. The basal metabolic rate of Wi on the twenty-eighth week (denoted by "T" in the graph) is probably too high since the patient had an oral temperature of 100.1° F. when the rate was determined. The question mark on the graph of thyroid dose for Pa. indicated that for a 2-week period the dose of thyroid was indeterminate, but probably small.

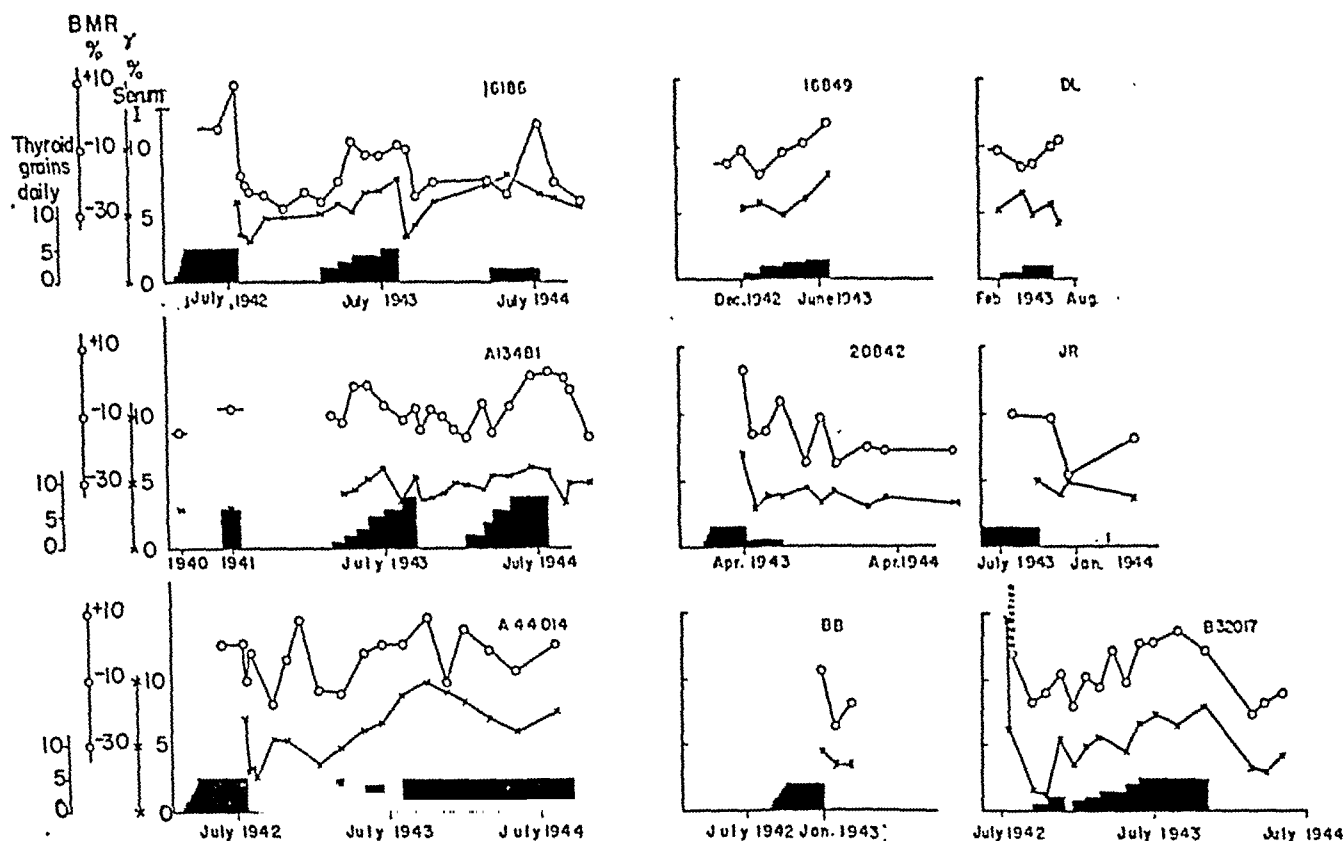


FIG. 2. BASAL METABOLIC RATE AND SERUM IODINE IN VARIOUS NONMYXEDEMATOUS SUBJECTS RECEIVING THYROID MEDICATION AT VARIOUS LEVELS OF DOSAGE FOR EXTENDED PERIODS OF TIME

In general, metabolic rate and serum iodine are well correlated with one another but poorly correlated with the level of thyroid dosage. The differences in response to thyroid among various subjects poorly should be noted.

The reactions to thyroid medication of the basal metabolism alone of the 3 subjects on the extreme left (16186, A13481, and A44014) have been described elsewhere and their case histories summarized there (2).

J. R. and B. B. were normal subjects who had been receiving thyroid for some time prior to this study solely because of initially low basal metabolic rates. Case 16849 and D. L. were normal subjects with an initial slight depression of metabolism who were deliberately placed on increasing doses of thyroid. Case 20842 underwent a subtotal thyroidectomy in 1942 which was followed by hypoparathyroidism and a low basal metabolic rate. At the time of the first observation by us (April, 1943), she had been on 3 grains of thyroid daily for some time, and was very nervous. Thyroid medication was first reduced to 1 grain a day and then omitted altogether. No clinical signs of thyroid deficiency ever developed. She was treated throughout with calcium and Hytakerol. Case B32017 represents a somewhat similar case followed from time of operation. Thyroid medication was given soon after operation because of definite symptoms of hypothyroidism. The dose was gradually raised to 5 grains daily, then stopped altogether. No symptoms of hypothyroidism developed, although both iodine and metabolism dropped.

symptoms of the patients during the period of excessive thyroid medication were those of a mild to moderate degree of hyperthyroidism, without exophthalmos or palpable enlargement of the thyroid gland.

With thyroid medication, the metabolic rates rose somewhat, but were not consistently maintained above +15 per cent until the dose was increased to 10 grains per day or more. With further increase in dosage, metabolic rates frequently rose above +30 per cent, but never exceeded +50 per cent. With discontinuance of thyroid

treatment the metabolic rates fell rapidly at first. Subsequently, however, the fall was much less rapid, so that the decrease in basal metabolic rate approximated the logarithmic decay curve noted by previous observers (8, 9).

The serum precipitable iodine values for the 4 patients during the initial 5-week control period were well within normal limits. The serum iodine tended to rise following thyroid administration. As with the basal metabolic rate, however, frankly abnormal levels (above 8.0 gamma per cent) were not consistently maintained until a dose of 10

grains per day was reached. Further increases in thyroid dose occasioned further increases in serum precipitable iodine (10). When thyroid treatment was discontinued the serum precipitable iodine fell so abruptly that within a single week normal or subnormal values were attained. By the end of the third or fourth week without thyroid, distinctly low values, comparable to those observed in untreated myxedema, were found. Thereafter the serum precipitable iodine again increased until normal levels were attained by the fifth week after cessation of medication.

The mean of 20 control determinations of serum filtrable iodine on the group of schizophrenic patients was 0.4 gamma per cent. The tendency of the serum filtrable iodine to rise when large doses of desiccated thyroid were administered is depicted in Figure 3. With cessation of treatment, the serum filtrable iodine rapidly reverted to a normal low level.

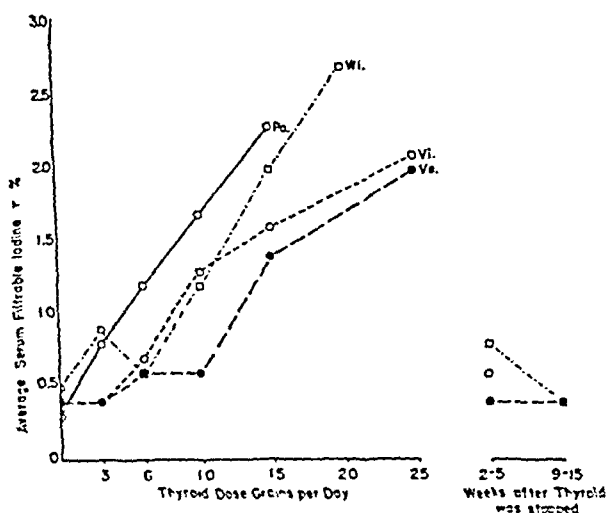


FIG. 3. THE EFFECT OF LARGE DOSES OF DESICCATED THYROID ON SERUM FILTRABLE IODINE IN THE EUTHYROID SCHIZOPHRENIC PATIENTS

Each point represents the average of all determinations for a given patient on a given dose of thyroid. The larger the dose, the higher in general is the filtrable iodine of serum. After 2 to 5 weeks concentrations of filtrable iodine had returned to normal.

From initial normal values the serum cholesterol of Vi., Ve., and Wi. fell during thyroid treatment. Pa. also had extremely low serum cholesterol values during treatment but unfortunately

a cholesterol determination was not obtained during the control period. The greatest decrease was occasioned by the change from 0 to 3 grains per day, larger doses having comparatively much less effect. When thyroid administration was discontinued, the serum cholesterol concentration returned to normal.

Serum total protein decreased in response to the administration of thyroid. When thyroid treatment was stopped the serum protein tended to rise slowly toward the original level.

The influence of thyroid feeding on body weight in this group of patients was remarkably variable. Wi. lost weight during the whole period of medication but had already been losing weight during the control period. Vi. maintained weight on 3 and 6 grains daily but lost weight on larger doses. Pa. and Ve. showed no striking weight changes during the entire period of thyroid treatment. With discontinuance of thyroid medication the body weight tended to rise. The increase was most striking in Ve. who gained 25 pounds in 6 weeks, attaining a level considerably above her initial weight. None of the patients would admit any change in appetite during or following the period of thyroid feeding.

The changes in heart rate roughly paralleled the changes in basal metabolic rate. On the average a change of 10 per cent in the metabolic rate was associated with a change of 5 beats per minute in the heart rate. During the period of thyroid feeding the pulse pressures of all 4 patients rose considerably. For patients Ve. and Vi., the rise was due to a simultaneous increase in systolic and decrease in diastolic pressures. For patient Pa., the pulse pressure rise was due almost entirely to a marked increase in systolic pressure, the diastolic pressure showing no significant trend. The reverse was true for patient Wi. In general there was a fairly high degree of correlation between the pulse pressure and pulse rate. Such minor fluctuations in body temperature as occurred during the study appeared to be due to random variation and could not be correlated with basal metabolic rate or dose of thyroid. The average oral temperature under basal conditions for all readings on all patients was very close to 98.0° F. Of 139 temperature readings only 8 fell outside the range of 97.0° F. to 99.0° F. The erythrocyte count and hemoglobin of Pa. and Ve. showed no significant variations during the course of the study. The other 2 patients developed a slight anemia during the period of thyroid feeding. Wi.'s hemoglobin dropped to an average of 11.6 grams per 100 ml. on 0 to 6 grains of thyroid per day, to an average of 10.4 grams per 100 ml. on 15 to 20 grains of thyroid per day. The erythrocyte

change for Vi. was from 12.9 grams per 100 ml. to 11.6 grams per 100 ml. The red cell count fell commensurately. Although the total leucocyte count of Wi., underwent considerable fluctuation during the experimental period, no definite trend was evident. For the other 3 patients a distinct drop in the total leucocyte count during thyroid medication occurred. The lowest total white counts for Ve., Pa., and Vi. were 3,300, 4,300 and 4,700 cells per cubic millimeter, respectively. This fall in total white count was due almost entirely to a decrease in the absolute number of neutrophils. Although there was a relative lymphocytosis, the absolute number of lymphocytes was remarkably constant. After thyroid was stopped the hemogram tended to return to normal.

B. New Haven Hospital Patients

Figure 2 illustrates the effects of smaller doses and smaller increments of dosage continued over longer periods of time than those used in the schizophrenic patients of Figure 1. Changes of serum iodine and of basal metabolism in 9 non-myxedematous patients on various levels of thyroid medication are charted. In general the results were quite similar to those shown in Figure 1. Basal metabolism followed serum iodine, while both were poorly correlated with the dose of thyroid in most subjects. Different subjects exhibited tolerance in varying degrees; 16186 failed to respond to 3 grains, but responded to 5 grains daily; A13481 tolerated 8 grains daily without significant change; 16849 tolerated only 2 grains daily and exhibited a distinct response to 3 grains. B32017 responded to 2 grains shortly after operation, while still partially hypothyroid; later 3 grains daily were tolerated but a sustained response followed a daily dosage of 5 grains. Fall of serum iodine to subnormal levels upon abrupt cessation of medication, followed by spontaneous recovery, is well illustrated in case 16186 (1942 and 1943). Under comparable circumstances serum iodine also fell and recovered less dramatically in case A13481, and fell in case A44014.

DISCUSSION

Except for exophthalmos and goiter, most features of naturally occurring Graves' disease were reproduced by the oral administration of dried thyroid in large doses. Basal metabolism and serum precipitable iodine were elevated, while serum cholesterol was depressed. The responses of body weight, heart rate, and blood pressure were

similar to those of spontaneous hyperthyroidism. The changes in total leucocyte count and in the relative proportion of lymphocytes in Graves' disease were reproduced by thyroid medication.

The effect on the basal metabolism of doses of thyroid below 6 or 8 grains per day was exceedingly capricious. For instance, the metabolic rate of Vi. (Figure 1) rose markedly on 3 grains of thyroid daily but failed to change significantly when the dose was doubled. The rate of A13481 was unaltered by a gradual increase of thyroid to 8 grains daily, while 16849 responded to an increment of dose as small as 1 grain daily. With increase of dosage above 10 grains daily the metabolic rate always rose somewhat above normal, but the relationship between dose and response was unpredictable and highly irregular. These observations confirm the experience of previous workers (2, 11, 12).

In contrast to the poor correlation between thyroid dose and metabolic rate was the excellent correlation under most circumstances between serum precipitable iodine and metabolic rate. A stable serum iodine was regularly associated with failure of response of basal metabolism to thyroid medication (Figure 2). Whenever the metabolic rate increased significantly, there was a parallel increase in serum iodine. Each point in Figure 4 represents 1 pair of values of those 2 variables derived from the 4 experiments of Figure 1. Values after discontinuance of thyroid medication have been excluded. The distribution is curvilinear. Assuming the relationship to be logarithmic, as indicated by the solid lines in the graph, the coefficients of correlation between the basal metabolic rate and the logarithm of the serum precipitable iodine were +0.93, +0.92, +0.88, and +0.81 for Pa., Ve., Vi., and Wi., respectively. This high degree of correlation indicates a close functional relationship, but by no means proves that the relationship is actually a logarithmic one. Indeed, if the same logarithmic relationship is assumed to extend below the normal range into the zone of values associated with hypothyroidism, the theoretical curve fails utterly to coincide with the observed values.

With increasing thyroid dosage, there was almost no lag of the rise in the basal metabolism behind that of the serum iodine. This is shown in

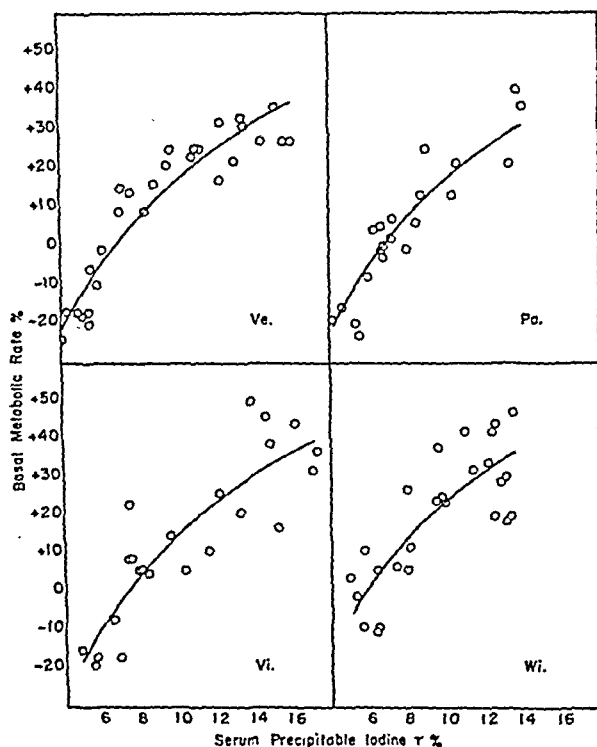


FIG. 4. CORRELATION BETWEEN SERUM PRECIPITABLE IODINE BASAL METABOLIC RATE IN FOUR EUTHYROID SCHIZOPHRENIC PATIENTS

The points represent individual determinations of serum precipitable iodine and corresponding values of basal metabolic rate. Values determined after thyroid was discontinued are omitted for reasons discussed in the text. The solid lines are the regression curves obtained when basal metabolic rate is assumed to be a function of the logarithm of the serum precipitable iodine.

Figure 5, in which changes in serum iodine and in metabolic rate of the 4 schizophrenic patients following an increase in thyroid dose have been plotted against time. The curves represent mean values for all doses. On the average, the serum iodine had almost attained its maximum level 1 week following a change in dose, while the basal metabolic rate required 2 weeks to reach its maximum level. This difference is of doubtful significance.

This apparent parallelism in Figure 5 may conceal a true lag in metabolic response behind increase in serum iodine concentration, since dosage was being progressively increased. With cessation of thyroid dosage, certainly, serum iodine fell much more abruptly than did the basal metabolic rate. In fact all correlation between the 2 vari-

ables was lost during the few weeks just after abrupt withdrawal of medication. Had these points been included in Figure 4, the correlation between serum iodine and basal metabolic rate would have been considerably less exact. Whereas the serum precipitable iodine of Wi. and Ve. fell to a low point 3 weeks following withdrawal of thyroid, the basal metabolic rates did not reach their lowest post medication values for 9 to 15 weeks (Figure 1). The serum iodine of Vi. dropped to a definitely subnormal value by the first week after interruption of medication, while the simultaneous basal metabolic rate was still +15 per cent (Figure 1). Decline of the serum iodine without comparable fall of the basal metabolic rate following sudden interruption of thyroid medication also appeared in cases 16186, A13481, and A44014 (Figure 2). A similar lag of the metabolic response behind the serum iodine

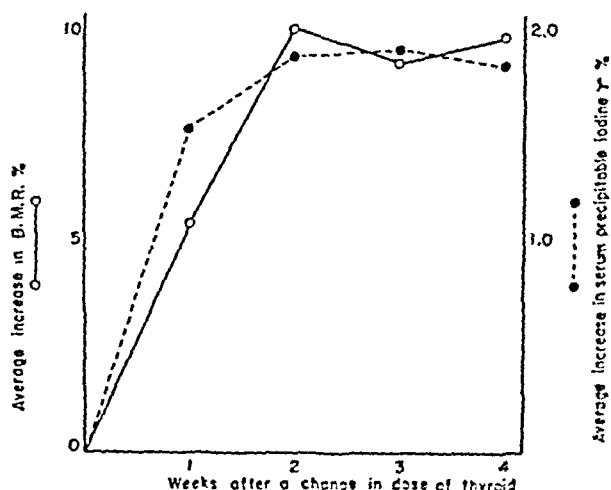


FIG. 5. PARALLELISM BETWEEN THE RATE OF CHANGE OF SERUM PRECIPITABLE IODINE AND OF BASAL METABOLIC RATE AFTER AN INCREASE IN THYROID DOSE

With increasing thyroid dosage there is almost no lag in the rise in the basal metabolism behind that of the serum iodine. This contrasts with the marked lag of basal metabolism behind serum iodine with abrupt discontinuance of thyroid medication (Figures 1 and 2).

The increase in serum precipitable iodine or in basal metabolic rate occasioned by an increase in the dose of thyroid has been plotted against the time elapsed since the change in dose. The origin corresponds to the last value obtained while the previous dose was being administered. The points represent average values for increasing thyroid dosage in all 4 schizophrenic patients and for all increments of dose.

concentration has been observed following thyroidectomy (patient B32017, and Figure 2 of the preceding paper) and following discontinuance of thyroid medication in patients with myxedema (1).

Presumably the lag of metabolic response behind serum iodine means that the thyroid hormone tends to disappear from the serum before it disappears from the tissues. The subnormal values of the serum iodine developing immediately after discontinuance of thyroid medication suggest not only inhibition by the medication of normal activity of the thyroid gland, but also a distinct delay in resuming its usual full activity. Persistence of an excessive rate of destruction of thyroid hormone may be a contributory factor.

Possible mechanisms of euthyroid tolerance to thyroid medication. In discussing possible reasons for the relative insensitivity of euthyroid subjects to desiccated thyroid, a group of workers (2) suggested that "the non-myxedematous subject possesses the ability, wanting in patients with myxedema, to inactivate thyroid substance and intravenous thyroxine." Perhaps it would have been more conservative to have suggested merely that this ability to inactivate thyroid hormone is very much greater in euthyroid subjects than in those with myxedema. The data of the present paper provide further support for this hypothesis, in that they disprove the existence of any decreased sensitivity of the tissues of the euthyroid subject to the thyroid hormone. The metabolic rate rises at least as much, for a given rise in serum iodine, in euthyroid as in myxedematous subjects (Figure 6). Of course a much larger dose of thyroid is required to produce a gross rise of serum iodine in the euthyroid subjects. The rise in the metabolic rate to supranormal levels in euthyroid subjects, induced by huge doses of desiccated thyroid was associated with a rise in serum iodine of about the same degree as that found in spontaneous hyperthyroidism of comparable severity. Whenever the metabolic rate was unaffected by thyroid medication, the serum iodine was also unchanged. It has been pointed out before (2) that impaired absorption of thyroid substance from the gastrointestinal tract by euthyroid subjects is an unsatisfactory explanation of their greater tolerance, since they

are also comparatively insensitive to thyroxine administered parenterally. Also difficulty in absorption is never seen either in spontaneous or induced myxedema, so that it is highly improbable that euthyroid subjects without intestinal defects are peculiar in their absorption of thyroid hormone. Some storage of the hormone in the thy-

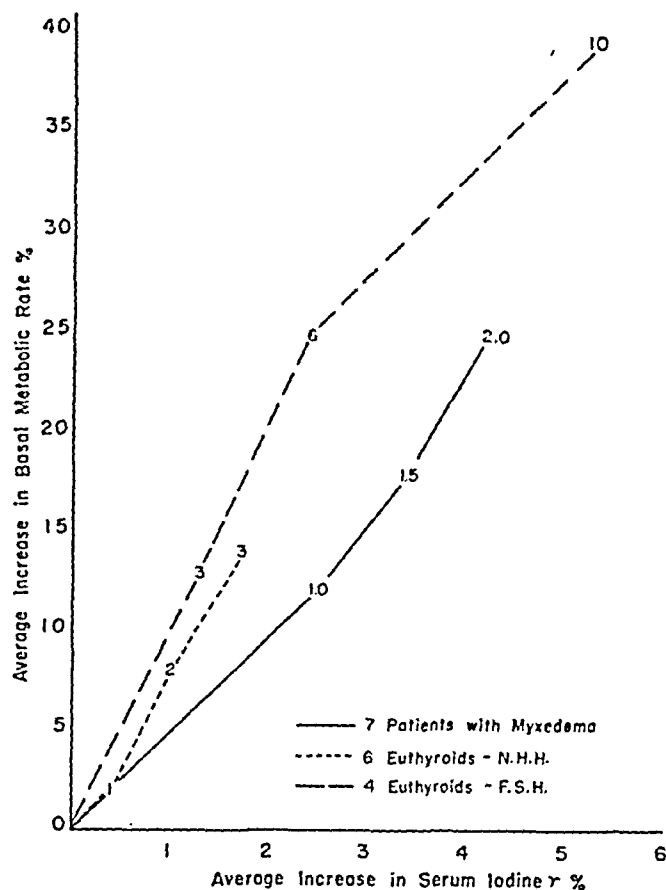


FIG. 6. RELATIONSHIP BETWEEN THE INCREASE OF SERUM PRECIPITABLE IODINE AND OF BASAL METABOLIC RATE OF MYXEDEMATOUS PATIENTS AND OF EUTHYROID SUBJECTS GIVEN DESICCATED THYROID

The increase in basal metabolic rate above the average premedication level is plotted against the corresponding increase in serum precipitable iodine at various levels of thyroid dosage. The dose in grains per day is indicated by the figures on the curves. The curve for the patients with myxedema was calculated from data reported in the preceding paper (1). Data from the 2 groups of euthyroid subjects reported here are plotted separately, the New Haven Hospital patients being indicated by short dashes, the Fairfield State Hospital patients by long dashes. Each point is the average for all patients in a given group on a given dose of thyroid.

Note that the basal metabolic rate rises at least as much per unit increase in serum iodine in the euthyroid subjects as in those with myxedema, but that a much larger dose of thyroid is required.

roid gland as colloid is possible, but does not explain the indefinite persistence of tolerance. Enlargement of the thyroid gland was never noted even after weeks of administration of huge doses of thyroid substance to the schizophrenic subjects, although the gland would have had to increase by several hundred grams had even half the administered hormone been stored. Extensive temporary storage in the thyroid gland or elsewhere is also improbable because a low serum iodine develops during the period following cessation of medication, and because subsequently there is never evidence of excessive release of stored hormone.

Inhibition of hormone production by the thyroid gland apparently did occur, but this factor only partially explains euthyroid tolerance to thyroid medication. There is good experimental evidence that the administration of thyroid hormone to intact animals depresses the normal activity of the thyroid gland (13, 14). In the present study of human subjects, the fall in serum iodine to very low levels when thyroid medication was abruptly withdrawn probably resulted from inhibition of normal production of hormone by the thyroid gland during the previous period of thyroid medication. Normal production of the hormone is, however, equivalent to not more than 3 grains of desiccated thyroid per day (1), so that complete cessation of endogenous production of hormone would account only for tolerance to this small daily dose. Were this the only mechanism concerned with the maintenance of tolerance, serum iodine and basal metabolism of all euthyroid subjects should respond to as much as 5 or 6 grains of thyroid daily. In point of fact, however, some normal subjects can tolerate as much as 8 grains daily without any significant alteration in serum iodine or metabolic rate (Figure 2).

A logical explanation of tolerance to amounts of desiccated thyroid in excess of 3 grains per day is that the normal thyroid gland can inactivate the exogenous hormone. This it might do by degrading active hormonal iodine to inorganic iodine, by a reversal of the reactions which, under normal circumstances, lead to the formation of hormone from inorganic iodine. The significant rise in the inorganic filtrable fraction of the serum iodine during massive thyroid feeding (Figures 1 and 3) would lend support to this hypothesis. It

is not certain that all the inactivation takes place in the thyroid gland. Any large measure of inactivation anywhere else, however, would imply a fundamental difference between the tissues of euthyroids and the tissues of myxedematous patients with respect to their ability to dispose of thyroid hormone, since the only known difference between adequately-treated myxedematous patients and euthyroid subjects is the presence of functional thyroid tissue in the latter.

There is suggestive evidence that tolerance to desiccated thyroid may require considerable time for full development. Many euthyroid patients experience a sharp rise in metabolic rate for a few weeks subsequent to an increase in thyroid dose, but if the dose is maintained for a longer period of time the metabolic rate tends to drift downward towards its original level (2). Any latency in the development of tolerance in the various patients described in this paper would be partially masked by the gradual step-wise manner in which the dose was increased. Three of the 4 schizophrenic patients, while on the highest dose of thyroid for 6 to 8 weeks, showed no clear-cut evidence of a decreasing response. However, for 2 of the patients there was some suggestion of a partial loss of tolerance during a lapse in thyroid treatment. Pa. had been started on 15 grains of thyroid per day, but after 1 week at this level the dosage was markedly reduced. Due to a confusion in orders, during the next 2 weeks she received irregularly an unknown, but probably small amount of thyroid. With resumption of a regular dose of 15 grains per day, both serum precipitable iodine and basal metabolic rate rose to levels much higher than would have been expected from this patient's previous responses. Similarly, when patient Wi. was replaced on a dose of 20 grains per day after a lapse of 2½ weeks, the serum iodine and metabolic rate both rose to a higher point than during the previous weeks on the same dose. These observations indicate the need for further investigation of the time necessary for the development of tolerance.

The tolerance of non-myxedematous patients to thyroid medication probably accounts for the apparent impunity with which large doses of dried thyroid may be administered under many circumstances. Whether permanent ill effects may re-

sult from long continued administration of thyroid substance to euthyroid subjects is not known. Our experiments have demonstrated only functional depression of the thyroid gland, while thyroid is being given and for a brief period after its withdrawal. This fact alone, however, suggests that long continuance of thyroid medication may not be wholly benign. On *a priori* grounds, caution is certainly indicated, especially in the continuous administration of thyroid to adolescents and to patients recovering from subtotal thyroidectomy.

SUMMARY AND CONCLUSIONS

1. Many euthyroid subjects are able to tolerate considerable amounts of desiccated thyroid without significant alteration in either serum precipitable iodine or basal metabolic rate.

2. The administration of larger quantities of dried thyroid to euthyroid subjects produces abnormal elevation of both serum iodine and basal metabolic rate and elicits most of the signs and symptoms of spontaneous hyperthyroidism, but the dose of thyroid required is usually much greater than is needed to produce an equivalent degree of hyperthyroidism in myxedematous patients.

3. When the administration of desiccated thyroid to euthyroid subjects is abruptly discontinued, the serum precipitable iodine frequently falls to abnormally low levels, probably indicating temporary inhibition of hormone production by the normal thyroid gland during, and for a brief period following, thyroid feeding.

4. Rapid and transient changes in serum precipitable iodine may occur without comparable fluctuations in basal metabolic rate. When alterations in the level of serum precipitable iodine are slower and more sustained, the lag in metabolic response is not as evident, and there is a high degree of correlation between the 2 variables.

5. The tissues of euthyroid subjects are at least as sensitive to thyroid hormone as are those of myxedematous subjects.

6. Degradation of active hormonal iodine to inorganic iodine by the normal thyroid gland is suggested as the most likely explanation of the tolerance of euthyroid subjects to amounts of desiccated thyroid in excess of 3 grains per day.

PROTOCOLS

Vc. was a 33-year-old white female with schizophrenia, paranoid type, of at least 2 years' duration. Although correctly oriented and in good contact with her surroundings, she believed herself persecuted by creatures half man and half beast. She was highly co-operative. Her mental symptoms were uninfluenced by thyroid medication. She was tall and thin without significant physical abnormalities except for vasomotor instability characterized by marked flushing of the skin of face, neck, and arms. Blood pressure was 100/60. On 6 grains of thyroid daily the vasomotor instability increased, and the patient fainted on 2 occasions. There was a slight tremor of the extended fingers. When larger amounts of thyroid were administered, the tremor became more pronounced and the vasomotor instability was further accentuated. The skin was warm and moist. The patient complained of fatigue and a "draggy" feeling. When thyroid was discontinued the tremor disappeared, the skin became drier, and the vasomotor instability decreased. Coincident with a marked gain in body weight, the patient became somewhat lethargic, but she never developed any signs or symptoms of myxedema.

Vi. was a 23-year-old white female with a 3-year history of schizophrenia, hebephrenic type. Before thyroid was administered she was extremely lethargic, withdrawn, and almost mute. She was hallucinated and grinned inappropriately. She was usually quite cooperative. During treatment with desiccated thyroid, the patient became more talkative and much less lethargic, assisting with ward work and taking long outdoor walks. With cessation of thyroid medication she reverted to her previous mute and disinterested condition. While this improvement in energy output may have been related to the induced hyperthyroidism, it should be noted that the patient had previously experienced similar, but apparently spontaneous, remissions. On physical examination prior to thyroid administration the patient was well developed and slightly obese with acne of the face and hirsutism of the face, back, and extremities. Blood pressure was 114/65. In response to thyroid, she developed tachycardia and a moist, warm skin with increased perspiration, but there was no definite tremor of the outstretched fingers. On 25 grains of thyroid daily, she complained of fatigue, and slight dyspnea on exertion. Observations during the post-medication period were interrupted when the patient was transferred to another hospital 5 weeks after thyroid feeding was stopped.

Wi. was a 54-year-old white female who had been hospitalized for 14 years because of schizophrenia, paranoid type, characterized by fixed delusions of grandeur. Although much deteriorated mentally, she was cooperative enough to serve as a subject in the present study. Her psychosis was unaffected by thyroid medication. The only abnormality found on physical examination prior to the administration of desiccated thyroid was slight narrowing, irregularity, and increased tortuosity of the retinal arteries. Blood pressure was 120/75. An x-ray of the chest was negative. On 6 grains of thyroid daily, the pa-

tient felt somewhat weak, and on 1 occasion had a fainting spell, with marked pallor of the face, and imperceptible pulse, but without loss of consciousness. On larger amounts of thyroid, a slight tremor of the extended fingers was noted, and on a few occasions there was transient ankle edema. After 7 weeks on 20 grains of thyroid daily the patient developed a fever of 102° F. The total leukocyte count was 11,200 with 73 per cent neutrophils. The pulse was 130 per minute. Except for a red and swollen but non-tender area at the base of 1 finger nail, there was no evidence of infection. Thyroid was discontinued. Despite the administration of sulfadiazine, the temperature continued to be somewhat elevated during the next 4 days. A chest x-ray showed left ventricular hypertrophy, but was negative for peripheral lung pathology. The only subjective complaint was stiffness and soreness of the shoulders, which persisted for some time after the fever had subsided. Although the cause of the hyperpyrexia was not clear, it was probably not due to the thyroid medication, since the same dose was later resumed for 2 weeks without untoward consequences.

Pa. was a 23-year-old white female who developed symptoms of schizophrenia, type undetermined, 3 years prior to admission. She was lethargic with brief periods of manic excitement, and claimed that she was infested by "bristle beetles." She was well oriented and quite cooperative, but resented hospitalization and made several attempts to escape. While thyroid was being administered, her comments and thinking appeared more coherent; this was thought to be only a temporary improvement unrelated to the thyroid medication. Physically she was well developed but thin and pale. Blood pressure was 100/60. Even on 15 grains of thyroid daily, tachycardia was the only physical sign of hyperthyroidism. Unfortunately the patient escaped from the hospital before the experimental program was completed.

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SERUM IODINE IN HYPOTHYROIDISM BEFORE AND DURING THYROID THERAPY^{1, 2}

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Many investigations have established the value of serum iodine concentration as a quantitative index of thyroid activity (1 to 7). Most previous reports have been concerned primarily with the increase in serum iodine which occurs in hyperthyroidism. The present study deals with the decrease in serum iodine characteristic of untreated hypothyroidism, and with the effect of thyroid therapy on the level of serum iodine of hypothyroid patients.

MATERIAL

Data are presented on 29 patients with spontaneous hypothyroidism and 1 patient (84883) with hypothyroidism following total thyroidectomy. Most of the patients were ambulatory. The majority have been studied in the Metabolism Clinic of the New Haven Hospital, many of them having been followed there for a period of several years. Two patients (F.S.H. 4774 and F.S.H. 4924) were observed at the Fairfield State Hospital. The remainder were private patients. In order to keep the group as homogeneous as possible, no patients have been included in whom the diagnosis of hypothyroidism was questionable or who developed hypothyroidism following subtotal thyroidectomy. In most of the patients included here the diagnosis of hypothyroidism was confirmed by striking improvement in signs and symptoms after a few weeks of thyroid therapy. In the few patients who remained untreated, or who were inadequately treated, the diagnosis rested on the presence of the clinical manifestations of hypothyroidism. Most of the patients did not present the extreme clinical picture usually described as typical of full-blown, classical myxedema. For example, generalized non-pitting edema was extremely rare. While most patients at some time during the course of their disease did exhibit some puffiness of the face, in a few even this slight degree of edema was never observed. It is reasonable to suppose, therefore, that many of the patients probably had small remnants of functional thy-

roid tissue which prevented them from developing full-blown myxedema. The left portion of Table I summarizes the clinical findings in these patients when the sign and symptoms of hypothyroidism were most outspoken. In certain patients thyroid therapy was stopped in order to confirm the need for its administration; such a procedure is indicated in the column "Days off Thyroid." It should be emphasized that the heart rates and basal metabolic rates recorded under the heading "Maximal Signs and Symptoms" are the lowest observed while the patient was receiving no thyroid. Since the basal metabolism could only be determined once or twice before thyroid therapy was begun, it is not certain that a completely "basal" level was always obtained. The average heart rates and basal metabolic rates off thyroid medication would tend to be somewhat higher.

Serum cholesterol and serum iodine from blood of patients in the post-absorptive state were determined by methods previously described (2, 8, 9, 10). The majority of iodine determinations were done on serum. Some figures, however, represent protein bound iodine as determined on serum proteins precipitated with zinc sulfate and sodium hydroxide (serum precipitable iodine) (2). Such values have been marked in the tables by the superscript P. Provided there has been no unusual intake of iodine, values for serum iodine and for serum precipitable iodine do not differ significantly. A few figures for serum iodine, indicated by the superscript B in the tables have been calculated from determinations of whole blood iodine by assuming that the cells contained no iodine and that the relative cell volume was 40 per cent (11). Barring unusual iodine intake, these assumptions are reasonably accurate, and the calculated values are comparable to values for serum iodine directly determined.

Since the amount of iodine in the serum of myxedematous patients is exceedingly small, some discussion of the accuracy of serum iodine determinations is necessary if the results are to be evaluated correctly. When duplicate 6 ml. aliquots of serum have been analyzed, the duplicates have usually checked within 0.5 gamma per cent. If the duplicates differed by more than 0.9 gamma per cent, the analysis was considered unsatisfactory and, if possible, was repeated. It is evident that if the concentration of serum iodine is below normal the possible analytical error represents a large proportion of the determined value. Hence, small differences in the results of determinations on the same patient at different times

¹ This investigation was aided by a grant from the Fluid Research Funds of the Yale University School of Medicine.

² A preliminary report of this work has appeared in the Proceedings of the American Society for Clinical Investigation in the Journal of Clinical Investigation, 1944, 23, 931.

TABLE I
Clinical and laboratory data in 30 cases of untreated hypothyroidism, and laboratory data on clinically adequate treatment

Patient Age	Untreated							Adequately treated				Remarks		
	Maximal signs and symptoms						Findings at time of initial iodine determination*			Laboratory findings*				
	Lowest B.M.R. per cent	Edema	Skin and hair	Voice	Retar- dation	Slowest heart rate	Days off thyroid	B.M.R. per cent	Iodine gamma per cent	Choles- terol mgm. per cent	Thy- roid grains per day		B.M.R. per cent	Iodine gamma per cent
23270 52	-14	++	++	++	+	52	54	-14	4.3u	315	2.0	-23	5.33	214
40152 52	-31	+	+	+	+	50	60+	-29	2.5u	427	1.5	-153	5.54	200
102366 54	-26	0	++	+	+	60	Life	-19	1.6	197				
89353 56	-34	+	+	+	+	72	Life 77 251	-34 -15 -6	2.0u 2.8 2.6	459 285 315	1.5	-23	5.33	2503
111116 58	-34	+	+	0	+	68	151	-27	1.2u	402	2.0	+4	5.43	1893
103359 57	-37	+	+	+	+	60	Life	-37	2.1	290	1.5	+5	5.7	177
43024 65	-27	+	++		++	60	49	-16	0.2	313	2.0	-44	5.43	1983
63345 56	-31	+	++	+	++	51	Life 43	-31 -23	2.1	335 229	1.0	-12	4.0	176
29117 57	-35	0	++	++	++	60	86	-31		518	2.0	-132	4.13	156
119116 64	-41	+	+	++	++	56	23	-26	1.2u	356	1.5	-13	4.73	245
104600 54	-35	+	+	+	+	81	Life	-26	0.5	911				
110223 54	-34		++	++	++	66	Life	-31	0.9	328	2.0	03	4.73	
119204 56	-35	+	+	++	++	54	Life	-18	0.6	381	2.0	-2	6.73	176
134 54	-48	0	+	+	++	52	11	-35		218	2.0	-123	3.73	
1360 59	-53	+	+	+	+	50	65	-19	0.8		1.0	-614	4.03	Grelin

TABLE I—Continued

Unit no. Age	Untreated										Adequately treated					Remarks
	Maximal signs and symptoms						Findings at time of initial iodine determination*				Thy- roid	Laboratory findings*				
	Lowest B.M.R.	Edema	Skin and hair	Voice	Retar- dation	Slowest heart rate	Days off thyroid	B.M.R.	Serum			B.M.R.	Serum			
									Iodine	Choles- terol			Iodine	Choles- terol		
	per cent							per cent	gamma per cent	mgm. per cent	per cent	gamma per cent	mgm. per cent			
A23925 52	-28	0	+	+	+	56	24+	-28	2.0	401		-18 [‡]	4.0	248		
A58944 56	-30	+	+	+	++	62	Life	-30		387						
A52821 69	-31	+	+	+	+	60	90	-31		550						
88453 23	-33	+	++	+	+	72	Life	-33	0.4 ^P	249		-10 [‡]	3.0 [‡]	216 [‡]		
FSH4774 62		+	++	+	+	60	Life		1.8 ^{P‡}	224		-4 [‡]	4.2 [‡]	145		
73340 80	-33	+	++	+	+	60	Life		1.2 ^P	67					Malnutrition. Patient died later, cancer of lung	
P-2 43	-26	+	++	+	+	62	Life		1.6 ^P	776					Malnutrition	
84883 70		+	++	+	+	64	Life	-26	0.8	393	3.0	-6 [‡]	5.8 [‡]			
B55424 53	-37	0	++	0		64	Life	-19 [†]	2.3 ^P	168					Hypothyroidism due to total thyroidectomy	
B56968 60	-19	+	++		++	60	Life	-37	0.9 ^P	156					Advanced pulmonary tuberculosis	
B57173 54	-39	+	+	+	+	50	Life	-19	0.6 ^P	331					B.M.R. -24 ten days after thyroid started. Malnutrition	
B38767 23	-30		+	+		64	Life	-39	0.2 ^P			-12	3.8 ^P			
B57274 49	-29	+	0			56	Life	-30	2.5 ^P	450						
B17780 50		0	+	+	+	60	Life		0.6 ^P	493					Non-toxic diffuse goiter	
FSH4924 59	-31	0	+	++	+	60	Life	-31	1.5 ^{P‡}						Marked improvement on thyroid	

* Superscripts indicate the number of determinations averaged.
† After three days of treatment.
‡ Calculated from whole blood analyses.

P Precipitable iodine.
M.M. Mendenhall

* Superscripts indicate the number of determinations averaged.
† After three days of treatment.
‡ Calculated from whole blood analyses.

P Precipitable iodine.
M Male. All other patients were females.

are of no significance, unless such differences are found consistently and repeatedly.

The basal metabolic rates were determined with the Benedict-Roth apparatus under the standard conditions described by Benedict, DuBois and others.

RESULTS

Serum iodine in untreated hypothyroidism. Serum iodine was determined in 26 of the patients before therapy with desiccated thyroid was begun, or after a lapse in treatment of at least 23 days' duration. With 1 exception (25270) all of the figures were well below the extreme normal range of 3.0 to 9.0 gamma per cent.³ Omitting this exception, the arithmetical mean was 1.3 gamma per cent, the range being from 0.2 to 2.5 gamma per cent. With the single exception noted above (25270) the basal metabolic rates were below -15 per cent when the serum iodine was determined (Table I). In 6 of the patients, however, the metabolism was but slightly substandard, from -16 to -19 per cent. This apparent discrepancy between the level of circulating thyroid hormone and the metabolic rate will be discussed below.

Serum iodine in hypothyroid patients adequately treated with desiccated thyroid. Nineteen of the patients were studied while taking an amount of desiccated thyroid sufficient to maintain them in what appeared clinically to be normal thyroid status (Table I). The maintenance dose requirement varied from 1 to 3 grains per day, and was most commonly 1½ or 2 grains per day. Under adequate treatment the serum iodine rose to normal, ranging from 3.0 to 6.7 gamma per cent and averaging 4.8 gamma per cent. In most of the patients the basal metabolic rate was also within normal limits at the time of the serum iodine determination.

The relationship between thyroid dose and serum iodine. Table II presents data on the serum iodine of all patients who were studied on at least 2 levels of medication. In a few cases it was possible to vary the dose experimentally in order to study the effect on serum iodine. In

³ Although most normal serum iodine values fall within the range of 4.0 to 8.0 gamma per cent, some patients without clinical evidence of thyroid disease have serum iodine concentrations as low as 3.0 or as high as 9.0 gamma per cent (12).

TABLE II
Serum iodine in hypothyroid subjects stabilized on various doses of U.S.P. thyroid

Unit no.	Concentration of iodine in serum of hypothyroid patients** x					Average* increase in serum iodine per grain of thyroid
	Grains of U.S.P. thyroid per day					
	0	1.0	1.5	2.0	3.0	
	gamma per cent	gamma per cent	gamma per cent	gamma per cent	gamma per cent	gamma per cent
A44182	2.5 ^B		5.5 ⁵	6.8		2.2
A38316	1.2 ^B	2.9 ²	4.0	5.4 ²		2.1
B25203	0.6	4.2 ²		6.7 ²		3.0
20800	0.8	4.0 ³		4.1		1.6
A23925	2.0	4.2 ²	5.2 ²	7.8		2.9
88453	0.4 ^P	2.9		3.7 ²		1.6
A49144	1.2 ^B		4.7 ²	3.7 ^B		1.2
84883**	0.6 ^P	3.4 ^P		4.9 ^P	6.1 ^{7P}	1.8
25270	4.3 ^B			5.3 ²		(0.5)
A94667	2.5 ²	5.4 ²	5.3 ²			1.9
B15259	2.1		5.7			2.4
43024	0.2			5.4 ²	6.9 ⁴	2.2
B10228	0.8			4.7 ²		2.0
A62475	2.1	4.0				1.9
B57173	0.6 ^P			3.8 ^P		1.6
B34400	0.5	2.0*				
B12566	1.6			4.3 ^{3x}		
29347		(6.5)		4.1 ²	3.5	
P-1				3.7 ²		
A58944		3.6 ²		4.0	6.1 ^{3x}	
A52821		3.0 ²				
Average (omitting 25270)						2.0±0.13

x Superscripts indicate number of determinations averaged.

* Calculated from minimum and maximum values.

** Determinations omitted in which dose was not stabilized for 2 weeks, except when otherwise indicated.

^B Calculated from whole blood analyses.

^P Precipitable iodine.

* On one grain only 7 days.

xx On two grains only 8 days.

xxx Value of 2.1 while on 3 grains omitted.

() Indicates doubtful value.

other patients the dose was changed from time to time as the clinical status of the patient demanded.

In 15 of the patients it has been possible to study the change in serum iodine as the dosage varied between 0 and one or more definite levels of thyroid dosage, each level of dosage having been maintained for at least 2 weeks prior to the iodine determination. For these patients values for the average increase in serum iodine per grain of thyroid have been calculated over the maximum range, from zero grains to the highest dosage level, neglecting any intermediate figures (Table II). The increase in serum iodine per grain of thyroid in hypothyroid patients was remarkably constant. The arithmetical mean was 2.0 gamma per cent

per grain of thyroid with a standard deviation of ± 0.5 gamma per cent and a standard error of the mean of ± 0.13 gamma per cent.⁴ One value of 0.5 gamma per cent (patient 25270) has been omitted from the average for reasons to be discussed presently.

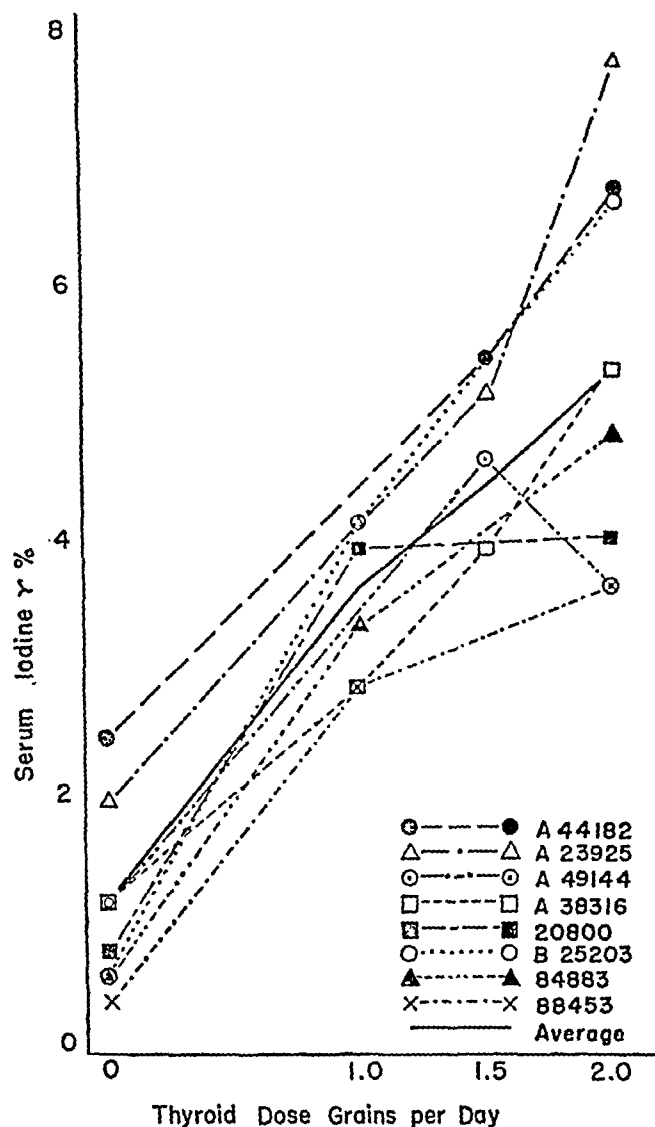


FIG. 1. AVERAGE SERUM IODINE CONCENTRATIONS IN THE 8 PATIENTS OF TABLE II

These patients were at one time or another maintained without thyroid, on a dosage of 2 grains daily and on at least 1 intermediate dosage. The data from the other 4 patients of Table II who were maintained on three levels of dosage (A94667, 43024, 29347, and A58944) are omitted since the lack of data at one or another of these 3 levels necessitated their exclusion in the calculation of the average.

$$^4 \text{ Standard deviation} = s = \sqrt{\frac{\sum (x - \bar{x})^2}{N - 1}}$$

$$\text{Standard error of the mean} = s_x = \frac{s}{\sqrt{N}}$$

The first 8 patients of Table II were studied while on 0 grains daily, 2 grains daily, and at least 1 intermediate dose of thyroid. In Figure 1, the serum iodine values for these patients have been plotted against the daily dose of thyroid. While there is considerable individual variation, the upward trend of serum iodine as the dose was increased is apparent. The solid line is drawn through the average values at 0, 1, 1½ and 2 grains. Not all patients were studied both on 1 grain and on 1½ grains of thyroid daily. In averaging the serum iodine values for these levels, therefore, it was necessary to calculate some figures by interpolation. The inclusion of these calculated figures necessarily tends to make the average curve artificially linear. Despite this slight bias, and despite the somewhat steeper slope of the average line from 0 to 1 grain per day than from 1 to 2 grains per day, the distribution of points suggests that the average curve cannot be reliably distinguished from a straight line.

Serum cholesterol values before and during treatment. Serum cholesterol was determined in 27 of these patients at a time when no thyroid was being administered (Table I). Twenty exhibited the hypercholesterolemia characteristic of hypothyroidism (13), the values ranging from 290 to 911 milligrams per cent. Of the 7 remaining patients with cholesterol levels below 265 milligrams per cent, 1 (P-1) had been off thyroid for only 11 days. There remain 6 patients with low or normal serum cholesterol values, the significance of which will be discussed later.

Serum cholesterol determinations were repeated in 13 of the patients after adequate treatment with desiccated thyroid had been instituted (Table I). Without exception, the hypercholesterolemia was abolished, the final values falling well within the normal range of 123 to 265 milligrams per cent (14).

DISCUSSION

Most of the early workers on blood iodine levels in hypothyroidism employed inadequate analytical techniques and their results are not considered here. Since the advent of more reliable methods for iodine estimation, a few reports of serum iodine in untreated hypothyroidism have appeared (Table III), but none of these have included any discussion of the effects of thyroid

TABLE III
Published values on serum iodine in hypothyroidism

Investigator	Number of cases	Serum iodine			Normal range	Comment
		Maximum	Minimum	Mean		
		gamma per cent	gamma per cent	gamma per cent		
Turner, DeLamater and Province (15)	5	9.7	1.5	5.8	6.7 to 16.7	Calculated from whole blood
Riggs, Gildea, Man and Peters (1)	7	2.8	0.5		4.0 to 7.0	Calculated from whole blood
Salter, Bassett and Sappington (4)	20	3.9	0.8	2.5	4.0 to 8.0	Serum precipitable iodine
Talbot, Butler, Saltzman and Rodriguez (6)	1			3.8	6.0 to 8.4	Serum precipitable iodine—adults
	4	3.0	1.8	2.3	4.0 to 7.0	Serum precipitable iodine—children
Present series	30	4.3	0.2	1.3*	3.0 to 9.0	

* Value of 4.3 excluded from mean. This omission is discussed in the text.

therapy on the level of serum iodine. With the exception of Turner and co-workers (15), all of these investigators have found that serum iodine values in untreated hypothyroidism are uniformly subnormal. This conclusion is abundantly confirmed by the series reported here (Table I). The 1 exception (25270) was a patient who had been on thyroid for 17 years and whose treatment was allowed to lapse in order to confirm the original diagnosis of hypothyroidism. After nearly 2 months without thyroid the patient experienced such a marked exacerbation of her symptoms as to necessitate resumption of therapy. At this time, however, the laboratory data were not in good accord with the clinical picture. The basal metabolic rate was only -14 per cent. The bound magnesium was 21 per cent of the serum total magnesium, well within normal limits (16). There was a slight hypercholesterolemia. In view of these findings it seems possible that the patient, fully aware of the efficacy of thyroid, may have resumed treatment herself a few days before her visit to the clinic. This would explain the normal iodine of 4.3 gamma per cent. An alternative explanation might be contamination of the blood sample with adventitious iodine before or during analysis, since the determination was not on the precipitated proteins. In any event, this single exception does not seriously challenge the conclusion that in untreated hypothyroidism, serum

iodine is characteristically subnormal, just as it is characteristically above normal in hyperthyroidism. Failure to find a low serum iodine in a patient suspected of having hypothyroidism strongly suggests that the patient's symptoms are not due to thyroid deficiency.

It should be emphasized that a single subnormal serum iodine value is not diagnostic of hypothyroidism. Equally small concentrations of iodine have been found in the blood of euthyroid subjects who have recently stopped taking large amounts of desiccated thyroid (17), and of hyperthyroid patients following subtotal thyroidectomy (12). By itself, therefore, a low serum iodine simply indicates undersecretion by the thyroid gland when the blood sample was collected, and it must be interpreted in the light of the patient's symptoms and previous history.

It has already been suggested that few of the patients reported here were completely devoid of functional thyroid tissue. However in 1 patient (84883) known to have had a total thyroidectomy, serum iodine values not significantly greater than 0 were observed. Furthermore, treatment of hyperthyroid patients with thiouracil or thiothrea in amounts sufficient to occasion symptoms of hypothyroidism may lower the concentration of serum precipitable iodine to the vanishing point (18). Total lack of thyroid secretion for a sufficient period of time would therefore appear to bring

virtual absence of circulating thyroid hormone, and grave doubt is cast on the existence in man of significant extrathyroidal manufacture of thyroid hormone. Of 17 patients in the present series who had been untreated for 1 year or more, 9 had serum iodine concentrations above 1.0 gamma per cent, *i.e.*, significantly greater than 0. The appearance of symptoms of hypothyroidism, therefore, does not necessarily imply complete absence of thyroid function.

The rise in serum iodine to normal levels when hypothyroid patients were treated with adequate doses of desiccated thyroid is in accordance with the well-established fact that relatively small daily doses of thyroid are sufficient to maintain subjects without thyroid tissue in normal thyroid balance. It also agrees with the hypothesis that, barring unusual sources of exogenous iodine, serum iodine is a reliable measure of the concentration of circulating thyroid hormone. Moreover, since the serum iodine levels in adequately-treated patients with hypothyroidism are no higher than in euthyroid individuals, the circulating hormone derived from substitution therapy with desiccated thyroid must be quantitatively as effective as the hormone produced by the normally active thyroid gland. It seems legitimate to conclude that the maintenance dose of thyroid required by any patient with hypothyroidism is that amount which will restore the serum iodine to normal.

The effect on serum iodine of administration of various amounts of desiccated thyroid has already been noted (Table II and Figure 1). Since the figures for increase in serum iodine per grain of desiccated thyroid were calculated from the difference between 2 iodine values, they were subject to twice the possible error of a single determination. In view of the large possible analytical error, the constancy of the increase in serum iodine per grain of desiccated thyroid is particularly remarkable. In obtaining the average value of 2.0 gamma per cent of iodine per grain of thyroid, the data on patient 25270 were omitted. As stated previously, there was reason to doubt the normal value obtained on this patient when she was supposedly receiving no thyroid.

The amount of hormone supplied by the normally-functioning thyroid gland has usually been considered as equivalent to the maintenance dose of desiccated thyroid for hypothyroid patients.

This hypothesis involves 3 assumptions: that all the dried thyroid is absorbed from the alimentary tract; that it is quantitatively as effective as the natural hormone; and lastly, that the hypothyroid patient's own gland contributes no hormone. Although complete intestinal absorption has not been proved directly, it is strongly suggested by the fact that in the treatment of human hypothyroidism, desiccated thyroid is even more effective than intravenous thyroxine in iodoequivalent amounts (19). The validity of the second assumption has already been discussed. The third assumption is not always justified since a patient with outspoken clinical hypothyroidism may have a serum iodine concentration of 1.0 to 2.0 gamma per cent. However, since each grain of dried thyroid causes an increase in serum iodine averaging 2.0 gamma per cent, even in the complete absence of endogenous hormone production, 3 grains of desiccated thyroid per day should result in a final serum iodine concentration of about 6.0 gamma per cent, a value close to the average normal level. The production of hormone by the normal thyroid gland may therefore be regarded as approximately equivalent, in terms of iodine content, to 3 grains of U.S.P. desiccated thyroid.

If each additional grain of thyroid administered to a hypothyroid subject causes a rise in serum iodine of about 2.0 gamma per cent, it is easy to understand why such patients are unable to tolerate more than 3 grains of thyroid daily without developing definite symptoms of thyroid excess such as nervousness, tremor, and tachycardia. If the initial serum iodine of thyroid were 1.3 gamma per cent (the average for this series of patients), and 4 grains of thyroid per day were administered, the serum iodine would presumably rise to about 9.3 gamma per cent, a value distinctly above the normal range. This calculation involves the unproven assumption that the linear relationship between dose and serum iodine would hold for doses greater than 2 grains per day. Nevertheless, the result of the calculation agrees with clinical observation that slight over-doses of thyroid are prone to induce high serum iodine levels and unpleasant symptoms of hyperthyroidism in previously hypothyroid subjects.

The average relationship between serum iodine and basal metabolic rate may be roughly evaluated. The effect of varying thyroid dose on the basal

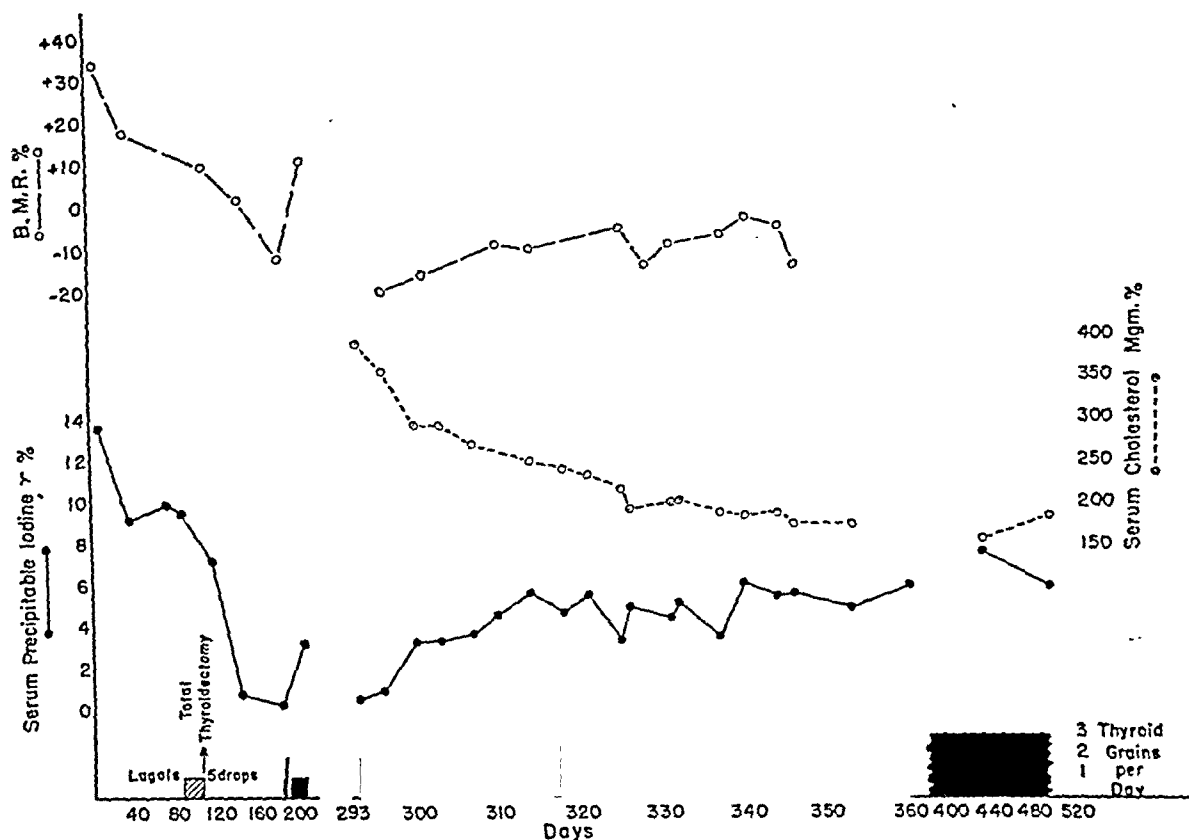


FIG. 2. CHANGES IN BASAL METABOLIC RATE, SERUM PRECIPITABLE IODINE AND SERUM CHOLESTEROL IN A HYPERTHYROID PATIENT SUBJECTED TO TOTAL THYROIDECTOMY

The elevated initial basal metabolism and serum iodine confirmed the diagnosis of moderately severe hyperthyroidism. Forty days after operation the serum iodine had already fallen to 1.0 gamma per cent, yet the metabolic rate was still +3 per cent. Thirty-eight days later the serum iodine was 0.5 gamma per cent—scarcely distinguishable from 0, while the patient was exhibiting signs and symptoms of hypothyroidism. Nevertheless the basal metabolic rate at this time had only decreased slightly, to -11 per cent. Following a brief course of thyroid therapy, the patient failed to return to the clinic for almost 3 months, during which time she took little or no thyroid. When seen again 6 months following operation the serum iodine was 0.8 gamma per cent and the serum cholesterol almost 400 mgm. per cent. Symptoms of hypothyroidism were outspoken. Unfortunately a determination of basal metabolism was not done until 3 days after treatment with thyroid was begun, at which time it was -19 per cent. The changes following the institution of therapy appear in the figure. Note the changes in the time-scale.

metabolic rate in hypothyroidism was analyzed and these workers (20) concluded that, for the range of 0 to 2 grains daily, the true dose-response curve was probably much closer to a straight line than to the curvilinear relationship suggested by other investigators (21). The former found that the basal metabolism increases about 12 to 16 per cent per grain of desiccated thyroid. Comparison with the slope of the average line of Figure 1 yields a value for the increase in basal metabolic rate of 6 or 8 per cent per gamma per cent increase in serum iodine. The relationship

holds only for values obtained after a given dosage had been maintained for several weeks without change. The metabolic rate responds much more slowly to alterations in thyroid status than does the serum iodine. In support of this statement, Figure 2 presents in detail the laboratory data on a hyperthyroid patient who developed hypothyroidism following total thyroidectomy. It is apparent that in this case the decrease in serum iodine following total extirpation of the thyroid gland was out of all proportion to the change in basal metabolism. In several additional cases the

basal metabolic rate has remained relatively high despite a definitely subnormal concentration of iodine in the blood serum. For example patient A94667, when first seen, had a basal metabolic rate of -34 per cent and a serum iodine of 2.0 gamma per cent. After more than a year of satisfactory treatment with thyroid, medication was discontinued for 251 days. During this time the basal metabolism never fell below -15 per cent, and after the 251 days without treatment was only -6 per cent. Yet serum iodine values of 2.8 gamma per cent and 2.6 gamma per cent were found 77 and 251 days after thyroid therapy was stopped. These values were almost as low as the initial one when the metabolic rate was frankly subnormal. Indeed, in the entire series of patients at the time of the initial iodine determination metabolic rates as low as -30 per cent or lower need not appear unless the patient had remained untreated for several years (Table I, columns 8 and 9). Yet in at least 3 instances (43024, A62475, and A49144) the serum iodine fell to low concentrations when thyroid had been omitted for less than 2 months. Similar dissociation between metabolic rate and blood iodine has been observed after subtotal thyroidectomy (12) and following discontinuance of thyroid medication in euthyroid subjects (17). It is not possible to state from our data exactly how rapidly the serum iodine decreases after thyroid has been stopped but it is clear that the decrease may take place weeks or even months before there is any marked change in the basal metabolism. Whether this lag is due to retention of active hormone in the tissues, or whether the fall in metabolism is dependent on slowly-occurring changes which are secondary to the diminished amount of active hormone in the body, cannot be stated until more is known concerning the mode of action of the thyroid hormone. In any event, it would appear that fluctuation in the level of serum iodine in hypothyroidism corresponds more nearly to the need of thyroid therapy than does the basal metabolic rate.

The previous reports in which some earlier articles were cited have emphasized the constancy of hypercholesterolemia in untreated hypothyroidism (13, 22). In the present series the incidence of normal or subnormal serum cholesterol values, in patients untreated for a reasonably long period

of time, was unusually high (24 per cent). Of the 6 patients who did not develop hypercholesterolemia (B12566, 88453, F.S.H. 4774, B55424, B56968, and 73340), 3 were suffering from severe malnutrition, 1 died soon afterwards with advanced hepatic insufficiency, 1 had active pulmonary tuberculosis, and 1 was without obvious complications. Both malnutrition and advanced hepatic insufficiency are known to be associated with depression of the serum cholesterol (23, 24, 25). Hypercholesterolemia is not, therefore, an obligatory accompaniment of hypothyroidism, and, in the presence of nutritional deficiency or advanced liver disease, the serum cholesterol level may be normal or even exceedingly low (73340).

SUMMARY AND CONCLUSIONS

1. In untreated hypothyroidism serum iodine is characteristically subnormal. In many hypothyroid patients, however, it is significantly greater than 0, indicating the presence of some functional thyroid tissue in such patients.
2. Desiccated thyroid in amounts sufficient to relieve the symptoms of hypothyroidism causes a return of serum iodine to normal levels.
3. On the average, a 1-grain increase in the daily dose of thyroid administered to hypothyroid subjects occasions an increase in serum iodine of 2.0 gamma per cent. The relationship between serum iodine and thyroid dose appears to be linear within the limits of 0 and 2 grains per day.
4. The basal metabolic rate responds much more slowly to alterations in thyroid status than does the serum iodine.
5. Evidence is presented in support of the hypothesis that, in terms of iodine content, the daily production of hormone by the normally functioning thyroid gland is roughly equivalent to 3 grains of U.S.P. desiccated thyroid.
6. It is concluded that serum iodine is not only a valuable aid in the diagnosis of hypothyroidism but is also a useful criterion of the adequacy of treatment with thyroid substance.

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TYPE SPECIFIC MENINGOCOCCIC AGGLUTININS: II—THE RELATIONSHIP OF TITERS TO THE COURSE OF THE DISEASE¹

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In the first paper of this series (1) a technic was described which affords a simple method of detecting type specific agglutinins in individuals suffering from meningococcic infection. Agglutinin titers over $1/100$ were regarded as significant of the presence of specific antibody, while those up to and including $1/100$ were considered within the normal range. If this test was to have practical application for diagnostic purposes, or for determining the type of the infecting organism in cases where meningococci were not cultured, it seemed essential to study the agglutinin titers in bacteriologically proved cases of meningococcic infection during the entire course of the disease to determine: (a) When are agglutinins found in diagnostically significant titers? (b) What types of agglutinins are found? (c) Do these correspond in prevalence with the type distribution of recently isolated strains? (d) Do the types of agglutinins in patients' serums correspond in type and valence with the infecting organism in cases where both the infecting organism and the patient's serum were available for study?

The meningococcic agglutinin content of 252 bacteriologically proved cases of meningococcic infection was studied from March, 1942 to March, 1945. Samples of serum were obtained from the same patient from 1 to 34 times at intervals of from 1 to 21 days, *i.e.*, during the acute, subacute, and convalescent phases of the disease. All the patients included in this study received one of the sulfonamide drugs and none received serum.

METHOD

The method used was that described in our previous paper (1). Briefly, all serums were tested for Group I, Group II and Group II-alpha agglutinins. The cultures used for the antigens were standard stock strain cultures of well-established specificity and antigenicity. Serum dilutions ranged from $1/50$ to $1/1600$. Each dilution was

increased twofold. The tests were incubated at 37° C. for 2 hours, centrifuged at high speed for 10 minutes and kept in the refrigerator overnight. Readings were made after centrifugation and also following overnight storage. The last tube showing definite (2 plus) clumping was considered indicative of the titer of the serum.

RESULTS

Agglutinins in significant titers were found, for 1 or more of the types of meningococci, in 188 of 252 (75 per cent) cases. Only 1 sample of blood was obtained in each of 149 cases, 2 samples were collected in each of 59 instances, and from 3 to 34 samples were taken from each of 47 patients. For purposes of analysis, the 506 samples of serum obtained from these patients were arbitrarily grouped according to the phase of illness during which they were obtained. All samples of serum collected from the first to the fifth day after the onset of the disease were considered as representative of the acute stage, those from the sixth to the tenth day as subacute stage, and those from the eleventh

TABLE I
Distribution of the titers of the same patients tested during the acute, subacute, and convalescent stages of their illness

Agglutination titer***	Stage of illness during which blood was withdrawn		
	Acute	Subacute	Convalescent
Less than 1/100	1 to 5 days 16	6 to 10 days 1*	11 to 90 days 1**
1/100 to 1/200	0	0	1
1/200 to 1/400	2	5	2
1/400 to 1/800	2	8	4
1/800 to 1/1600	3	6	12
1/1600 and over	1	4	4
Total	24	24	24

* Serum from this patient showed a titer of 1/256 during convalescent stage.

** Serum from this patient showed a titer of 1/1600 during the subacute stage.

*** 13 patients tested using dilution range of 1/64 to 1/1024, 11 patients tested using dilution range of 1/50 to 1/1600 (1).

¹ Presented at the New York Academy of Medicine Clinical Research Meeting, May 16, 1945.

TABLE II
Protocols of individual cases of proved meningococcic infection

Case number	Diagnosis	Smear	Culture	Onset	Day after onset	Agglutination titer		
						Group I	Group II-a	Group II
1-	Meningococcic meningitis	Positive	No growth	5/26/43	1, 2	1/ 64	0	0
					3	1/ 128	0	0
					6	1/ 256	0	0
					7	1/ 512	0	0
					8, 9	1/1024	0	0
					10, 12	1/1024+	0	0
2-	Meningococcic meningitis	Positive	Positive I-II-a	9/26/43	2	0	0	0
					8, 10	1/ 800	0	0
					12	1/1600	0	0
					13	1/1600	1/100	0
					17	1/1600	1/400	0
					24	1/1600	1/200	0
					27	1/1600	0	0
					33	1/ 200	0	0
3-	Meningococcic meningitis	Positive	No growth	5/15/43	4	1/ 32	0	0
					5	1/ 64	0	0
					6	1/ 128	0	0
					7	1/ 256	0	0
					8 to 15	1/ 512	0	0
					18	1/ 256	0	0
					19, 20	1/1024	0	0
					21 to 28	1/ 512	0	0
					30, 32	1/ 256	0	0
					33	1/ 512	0	0
					34 to 37, 40, 42, 51	1/ 256	0	0
					59	1/ 128	0	0
					66	1/ 32	0	0
4-	Influenzal meningitis (control)	Positive	Positive Type B	11/6/43	5, 11, 16	0	0	0
5-	Pneumococcic meningitis (control)	Positive	Positive Type 27	11/8/43	7, 12, 16	0	0	0

to the ninetieth day as convalescent stage. In 155 instances where more than 1 sample of serum was obtained during a given period, an arithmetic average of the reciprocals of the titers was used, and the 2 or more specimens were considered as a unit. Only 68 of the 149 (46 per cent) serums collected during the acute stage were positive, while 84 of 106 (79 per cent) serums collected during the subacute stage were positive, and 49 of 72 (68 per cent) serums collected during the convalescent phase were positive. The time of onset was unknown in 24 instances. In this group, 18 (75 per cent) serums showed agglutinins in significant titers.

To clarify this problem further, a group of 24 patients was tested 1 or more times successively during each of the acute, subacute and convalescent phases. In instances where more than 1 sample of serum was tested during any one of

these periods, the titers were averaged using the method previously mentioned. The distribution of the agglutinin titers of these cases is given in Table I. Sixteen of the 24 cases showed negative agglutination titers during the acute stage. Only 1 case remained negative during the subacute stage but became positive during the convalescent stage. Another case that was positive during the subacute phase became negative during the convalescent phase. The increase in the number of patients showing agglutinins in titers above $1/400$ was also marked during the subacute and convalescent phases. In fact, while only 6 cases showed agglutinins over $1/400$ during the acute phase, this number was increased to 18 during the subacute and to 20 during the convalescent stage.

Table I shows the general trend of the group as a whole. The variation which was encountered in individual cases can best be shown by the fol-

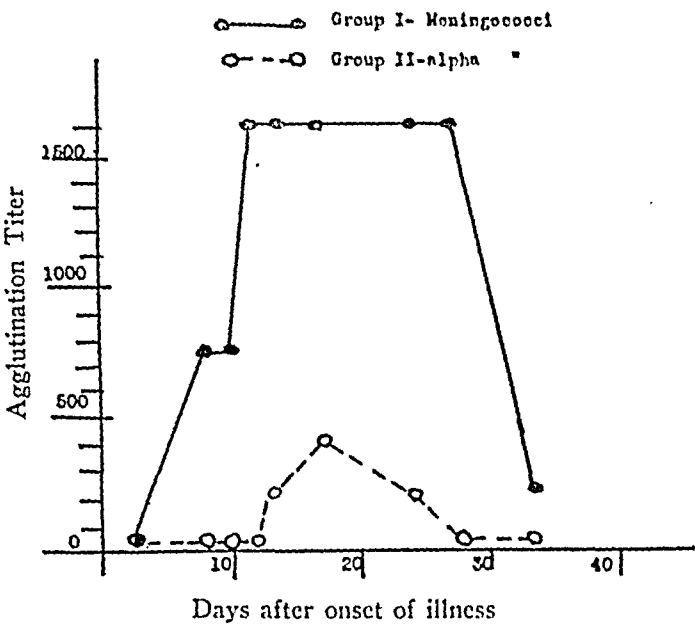


FIG. 1. AGGLUTINATION TITERS FOR GROUP I AND GROUP II-ALPHA MENINGOCOCCI ON SERUMS OBTAINED FROM PATIENT C. C.
Onset of illness 9/26/43.

tocols of typical cases. Three such cases and 2 control cases are presented in Table II. Case number 1 showed a gradual rise in agglutinin titer during the first 12 days of illness. In case number 2 agglutinins for Group I meningococci persisted until the thirty-third day. In this same instance, agglutinins for Group II-alpha meningococci made a delayed appearance and were observed from the thirteenth to the twenty-fourth day. Finally, in case number 3, samples of serum were taken at almost daily intervals during the entire course of the disease. This case showed a gradual

rise in titer during the first 3 weeks, and a gradual decrease in titer from the fifth to the eighth week. The final sample of serum taken 9 weeks after the onset of illness was negative. Case numbers 4 and 5 were representative of a group of 78 control patients, suffering from other forms of meningitis not caused by the meningococcus. Serums collected from these patients at comparable periods to the meningococcic infection cases were negative.

It is of interest to note that essentially the same rise and fall of agglutinin titers was observed in another group of serums collected from 128 clinically typical cases of meningococcic infection, without bacteriological confirmation of the causative agent. This will be the subject of a future report.

These results indicate that the curve of agglutinin production in cases of meningococcic infection was similar to the classical one of other febrile diseases such as typhoid fever (2). Typical curves of agglutinin titers in instances where tests were made on samples collected during the various phases of the diseases are presented in Figures 1 and 2.

In certain instances, the significance of the test was obscured by the fact that the beginning of the infection probably preceded the onset of clinical symptoms by a sufficiently long interval to allow for the formation of agglutinins in significant titers. Case numbers 1 and 2 in Table III represent 17 such instances in a group of 47 cases where 3 or more samples of serum were studied. Unfortunately, serums were not obtained in these 17 instances late in convalescence to determine a

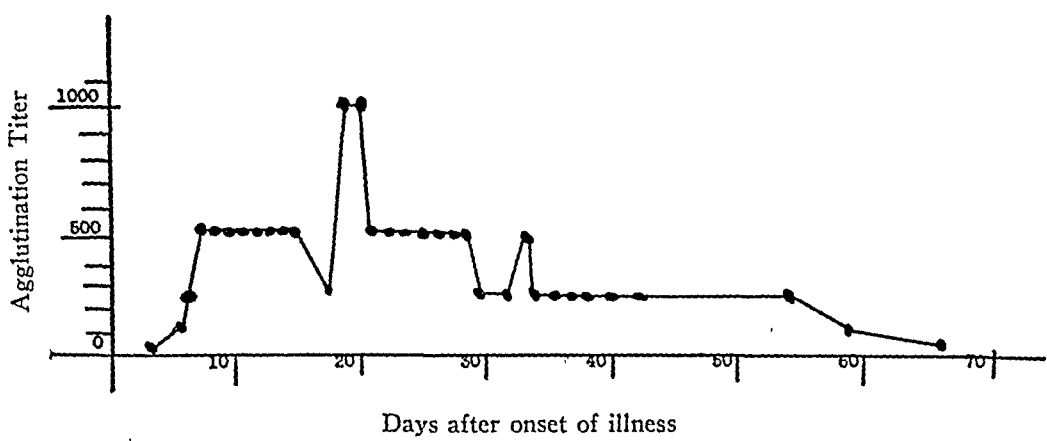


FIG. 2. AGGLUTINATION TITERS FOR GROUP I MENINGOCOCCI ON SERUMS OBTAINED FROM PATIENT C. C.
Onset of illness 9/15/43.

TABLE III

Protocols of individual cases of proved meningococcal infection showing significant agglutinin titers for type specific meningococci on the day of onset of the disease.

Case number	Diagnosis	Smear	Culture	Onset	Day after onset	Agglutination titer		
						Group I	Group II-a	Group II
1*	Meningococcal meningitis with meningococcemia	Positive	Positive I-II-a	6/18/43	1	1/1024	1/ 512	0
					3	1/ 512	1/1024	0
					6	1/1024	1/1024	0
					15	1/1024	1/ 256	0
2†	Meningococcal meningitis	Positive	No growth	5/23/43	1	1/ 256	0	0
					4	1/ 32	0	0
					8	1/ 64	0	0
					14	1/ 128	0	0
					16	1/ 32	0	0
					19	1/ 512	1/ 256	0

* 13 instances in a group of 47 cases showed similar high titers on the day of onset.

† 4 instances in a group of 47 cases showed high titers on the day of onset, accompanied by fluctuating titers on the several samples tested.

change in titer. Case number 2 was also typical of a group of 4 instances which showed a fluctuation of agglutinin titers during the first 3 weeks of illness.

Our own studies, as well as those of other observers (3 to 9), showed that Group I meningococci were the predominating type during the period of this investigation. Since March, 1942, we have classified 347 gram negative diplococci, isolated by this laboratory from samples of spinal fluid or blood. They were typed by the tube agglutination method, using type specific monovalent rabbit antisera. The typing sera were prepared from the same strains that have been

used as antigens in testing the agglutinin content of the patient's sera, and did not show cross reactivity between the different groups of meningococci when tested with strains of known antigenicity. Two hundred and ninety-five (85 per cent) were typed as Group I; 43 (12.5 per cent) as Group I-II-alpha; 1 (0.3 per cent) as Group II; 4 (1.2 per cent) as Group II-alpha; and 3 (0.9 per cent) could not be classified serologically, although on the basis of fermentation reactions, they belonged to the *N. meningitidis* group.

The type distribution of the agglutinins found in the patients' sera was very similar to the type distribution of the recently-isolated strains.

TABLE IV

Type distribution of meningococcal agglutinins in bacteriologically proved cases of meningococcal infection

Agglutinins in titers above 1/100 for group	Stage of infection during which blood was withdrawn								Total	
	Acute		Subacute		Convalescent		Onset unknown			
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
	1 to 5 days		6 to 10 days		11 to 60 days					
Monovalent serums										
I	52	72.2	49	87.7	29	76.3	15	66.7	142	75.6
II	0	0	0	0	1	2.7	1	5.5	2	1.1
II-alpha	0	0	0	0	0	0	0	0	0	0
Polyvalent serums										
I and II	1	1.4	1	1.7	0	0	0	0	2	1.1
I and II-alpha	17	23.6	8	13.3	6	15.7	5	27.8	36	19.0
I, II, and II-alpha	2	2.8	2	3.3	2	5.3	0	0	6	3.3
Total	72	100.0	60	100.0	38	100.0	18	100.0	188	100.0
Total Group I alone or in com- bination with other groups	72	100.0	60	100.0	37	97.3	17	94.3	186	93.2

These results are presented in Table IV. One hundred and forty-two of the 188 cases showing agglutinins in significant titers, produced agglutinins for Group I meningococci alone. Two cases showed agglutinins for Group II alone. The remaining 44 cases showed serums with a broader valence. Agglutinins for Group II-alpha, as well as for Group I, were observed in 36 instances; agglutinins for Group I and Group II in 2 cases; and agglutinins for Group I, Group II and Group II-alpha in 6 instances. The valence of the serum did not appear to have any relation to the phase of the disease during which the serum was collected. Serums with a broad valence were found in 20 of 72 (28 per cent) cases during the acute phase; in 11 of 60 (17 per cent) cases during the subacute phase; and in 8 of 38 (18 per cent) cases tested during the convalescent phase. They were also found in 5 of 18 (28 per cent) cases in which the date of onset was unknown. Furthermore, in 10 of 21 instances, in which successive samples of serum from the same patient were tested, Group II-alpha agglutinins were not evident in the first sample of serum tested, but were present in subsequent ones taken either late in the first week or during the second week of illness. The protocols of case number 2 in Table II and number 2 in Table III are typical of such findings.

The infecting organisms were available for classification in 82 of the 252 cases included in this study. Agglutinins in significant titers were observed in 59 of these patients, while the remaining 23 yielded negative results. Twenty of the 23 patients whose serums failed to show agglutinins were tested only once. These included 12 patients

tested during the acute stage, 3 tested during the subacute stage, 1 tested during the convalescent stage, and 4 instances with time of onset unknown. Three cases were tested during the acute and subacute phases, but not during the convalescent phase. Table V shows that 37 of the 44 strains which reacted with only 1 type of monovalent rabbit antiserum elicited only 1 type of agglutinins in the patient's serum, while 7 strains produced a serum with a broader valence. On the other hand, 12 of 15 organisms which reacted with 2 or more types of monovalent rabbit antiserum evoked agglutinins for more than 1 type of meningococci. No organisms in this series were typed as either Group II or Group II-alpha alone. These results indicate that there is a correlation between the infecting organism and the antibodies produced in the patient's serum.

DISCUSSION

The significance of finding type specific meningococcic agglutinins in human serums in the past was obscured by the antibodies introduced, in the majority of cases, when therapeutic antiserum was used. The use of chemotherapy alone, however, has made it possible to study the antibodies produced by the infecting organism itself.

The results of this study show that the type specific agglutinin production in cases of bacteriologically proved meningococcic infection followed the classical curve of other febrile diseases. The titers varied from $1/200$ to $1/1600$. Agglutinins were observed more regularly during the subacute than during the acute phase and reached their maximum titers during the early part of the convales-

TABLE V

Comparison of the group of the infecting organism with the group of the agglutinins found in the patient's serum

Infecting organism classified as group	Total number of cases	Total number agglutination titer		Group agglutinins found in patient's serum					
		<i>1/100 or less</i>	<i>above 1/100</i>	I	II	II-alpha	I and II	I and II-alpha	I, II, and II-alpha
I	65	21	44	37	0	0	1	5	1
II	0	0	0	0	0	0	0	0	0
II-alpha	0	0	0	0	0	0	0	0	0
I and II	0	0	0	0	0	0	0	0	0
I and II-alpha	16	2	14	2	0	0	0	10	2
I, II, and II-alpha	1	0	1	1	0	0	0	0	0
Total	82	23	59	40	0	0	1	15	3

cent phase. There was, however, considerable variation in individual cases, both as to the time at which the agglutinins were first observed and their persistence in the patient's serum. These observations demonstrate the importance of testing successive specimens from the same patient, especially during the first 2 or 3 weeks of illness. As recovery progressed, there was a gradual decrease in the agglutinin titer to pre-infection levels.

Agglutinins for Group I, Group II and Group II-alpha were found alone and in combination. Agglutinins for Group I organisms, which were the prevalent type in the outbreak studied were found in 99 per cent of the serums. There was also correlation between the infecting organism and its homologous human serum in cases where both the infecting organism and the serum were available for study. This indicated the validity of using the agglutination test, in titers above $\frac{1}{100}$, as a means of determining the type of the infecting organism in cases where meningococci were not cultured from the spinal fluid or blood and in which the "quellung" test was unsuccessful. The test may also be useful as an additional diagnostic procedure in cases of suspected meningococcal infection where other laboratory confirmation is lacking. In this connection, the importance of performing several tests to demonstrate the development of the change of agglutinin titers cannot be overemphasized.

An exact explanation for finding polyvalent human serums cannot be given on the basis of the facts on hand. Since all the usual tests have been applied to insure the specificity and antigenicity of the strains used as test antigens, it would not appear that these cross reactions are due to unsuitable and nonspecific antigens. There is a suggestion that it may be due to the valence of the infecting organism or to the remote possibility of 2 separate infections by 2 different groups of meningococci.

It has been apparent that the meningococci isolated during the period of this investigation had a high degree of antigenicity. This has probably been one of the factors in the demonstration of type specific agglutinins. Variation in antigenicity, as well as the different techniques employed, may account for the confusing results obtained by different investigators in the past.

A group of workers (10) recently reported essentially similar results in a group of 47 bacteriologically proved cases of meningococcal infection, although they obtained a lower range of agglutinin titers. It should be noted that these investigators used freshly-isolated strains and a slightly different technic for their agglutination test. In our original paper (1), we compared several methods of incubation, which included a centrifugation method similar to the one employed by Dowling and his collaborators. We pointed out in that report that immediate centrifugation did not appreciably alter the titer of the serum.

The usefulness and the limitations of this test as a diagnostic procedure are those of all other serologic tests. In the absence of clinical evidence, it is not possible to distinguish between active and latent infection. We showed (1) that agglutinins in significant titers were found in healthy hospital and laboratory personnel in repeated contact with the infection. In these instances, no attempt was made to correlate these findings with the carrier state. However, in 1935, one worker (11) was able to demonstrate agglutinins in the serums of carriers and felt that this condition could be looked on as an infection. In this connection we should like to mention also the finding of an apparent source of false positives in a group of 11 of 46 instances of gonococcal infection. The significance of this observation merits further study.

SUMMARY AND CONCLUSIONS

1. Agglutinins were found in diagnostically significant titers in the serums of bacteriologically proved cases of meningococcal infection during the acute, subacute, and convalescent phases.

2. There was considerable variation in individual cases both as to the time at which the agglutinins first appeared in significant titers and their persistence in the patient's serum. Titers fell to pre-infection levels between the third week and fourth month after onset.

3. Agglutinins were found for Group I, Group II, and Group II-alpha meningococci alone and in combination with each other.

4. The agglutinins corresponded in prevalence with the type distribution of the infecting organisms.

5. The type of the agglutinins found in the patients' serums corresponded with its homologous infecting organism in all cases where both the organism and the serum were available for study.

6. The agglutination test may be useful in titers above $\frac{1}{100}$ in determining the type of the infecting organism in cases where meningococci are not cultured from the blood or spinal fluid and in which the "quellung" test is unsuccessful.

7. Excepting diseases due to the gonococcus, this test may be of value as an additional diagnostic procedure in cases of suspected meningococcic infection where other laboratory confirmation is lacking.

The technical assistance of Miss Diana Blitz in the execution of this work is acknowledged with thanks. We are also indebted to Miss Ruth Gosling, Miss Helen Ackermann, and Miss Marie Romano for cooperation in obtaining the specimens of blood and for the isolation of the meningococcus cultures.

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HEMODYNAMIC ALTERATIONS IN NORMOTENSIVE AND HYPERTENSIVE SUBJECTS DURING THE PYROGENIC REACTION

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It is well known that toxic substances, probably derivatives of bacterial protein (1), frequently appear in improperly distilled water. A dramatic and often potentially dangerous syndrome of chill and fever (pyrogenic reaction), occasionally complicated by peripheral circulatory collapse, follows the intravenous administration of solutions contaminated with these substances. A marked increase in renal blood flow (2), a reduction in arterial pressure (3) and an increase in cardiac output (4) have been observed during the pyrogenic reaction in normal man, indicating a marked reduction in over-all peripheral resistance. Three stages of arterial pressure change have been described (3): namely, (a) a short period of elevated pressure during the chill and initial rise in temperature, (b) a phase characterized by a moderate fall in blood pressure, coinciding approximately with the appearance of renal hyperemia, and (c) a marked reduction in blood pressure with a narrowing of the pulse pressure some 4 or 5 hours after the administration of the pyrogen. Although antipyretics, such as amidopyrine, when given in adequate doses prior to injection of the pyrogen, will prevent chill and fever, they do not affect renal hyperemia or phases (b) and (c). The reduction of arterial pressure (c) is usually greater among hypertensive than among normal subjects and may be so profound as to result in a shocklike state, even in the absence of fever. It is not known whether the late reduction in blood pressure is a result of widespread vasodilation, reduced cardiac output or a combination of the two. Nor is it known whether there is any fundamental difference in response on the part of hypertensive as compared with normotensive individuals. The present study was undertaken to evaluate the hemodynamic factors involved in this reduction.

METHODS

Eight subjects, 6 of whom had essential hypertension, selected from the wards of the Third (New York University) Medical Division of Bellevue Hospital, and Evans Memorial Hospital (Boston University School of Medicine), were examined under basal conditions after several days of bed rest. All subjects were free of gross cardiac pathology.

Amidopyrine in doses of 0.6 gram was given to each subject every 4 hours for 5 or 6 doses during the 24 hours immediately prior to the administration of pyrogen. Typhoid vaccine and contaminated inulin (Pfanstiehl inulin lot No. 268) were used as sources of pyrogen. Pyrogenic inulin, in doses of 100 to 200 mgm. dissolved in sterile saline and passed through a Seitz filter, was administered intravenously to 4 of the hypertensive subjects (A. B., B. S., M. N., H. H.) while typhoid vaccine, in doses of 0.1 ml. (100 million organisms) was administered to 2 others (M. P., G. O.). Two normotensive subjects (P. H., S. V.) were given 300 mgm. and 150 mgm. of pyrogenic inulin, respectively. Control measurements were made prior to or within 20 minutes after the injection of pyrogen.

Cardiac output was measured with a modified Starr ballistocardiograph (5) using the wave area formula (6). Since ballistocardiograph complexes are abnormal in the majority of hypertensive subjects, only the results for subjects with normal complexes are reported in this study. All values for stroke volume and cardiac output have been corrected by the 18.5 per cent correction factor by which ballistocardiographic values have been shown to differ from those obtained by the direct Fick method (7). In 2 subjects (S. V., H. H.) cardiac output was determined by the direct Fick method following catheterization of the right auricle (8).

Arterial pressure in the femoral or brachial artery was recorded by arterial puncture and the Hamilton manometer (9) and mean pressure was computed planimetrically. A formula derived from Poiseuille's Law, based upon the output of the heart and the mean arterial pressure, was used in calculating the peripheral vascular resistance in absolute units (10).

Simultaneous renal function studies were carried out on 6 subjects (P. H., M. N., B. S., A. B., H. H., M. P.). Renal plasma flow was measured as the constant α β gamma amino dipyrate clearance (11, 12), and glomerular filtration rate as the mannitol or inulin clearance (12, 13, 14).

RESULTS

Normotensive subjects

Circulatory adjustments during the afebrile pyrogenic reaction were studied in 2 normal convalescent male subjects (P. H., S. V.). Figure 1 illustrates the immediate response to pyrogenic inulin (300 mgm.) seen in 1 of these individuals (P. H.). Peripheral resistance (R) decreased as renal plasma flow (C_D) and cardiac output (CI—

ballistocardiographic) increased. The output of the heart increased as a result of increases in both stroke volume (SV) and heart rate (HR). Arterial pressure (BP) showed a tendency to fall with a narrowing of the pulse pressure and at the termination of the study was definitely below the control level. No further depression of the blood pressure was noted and the subject was up and about several hours later without complaint.

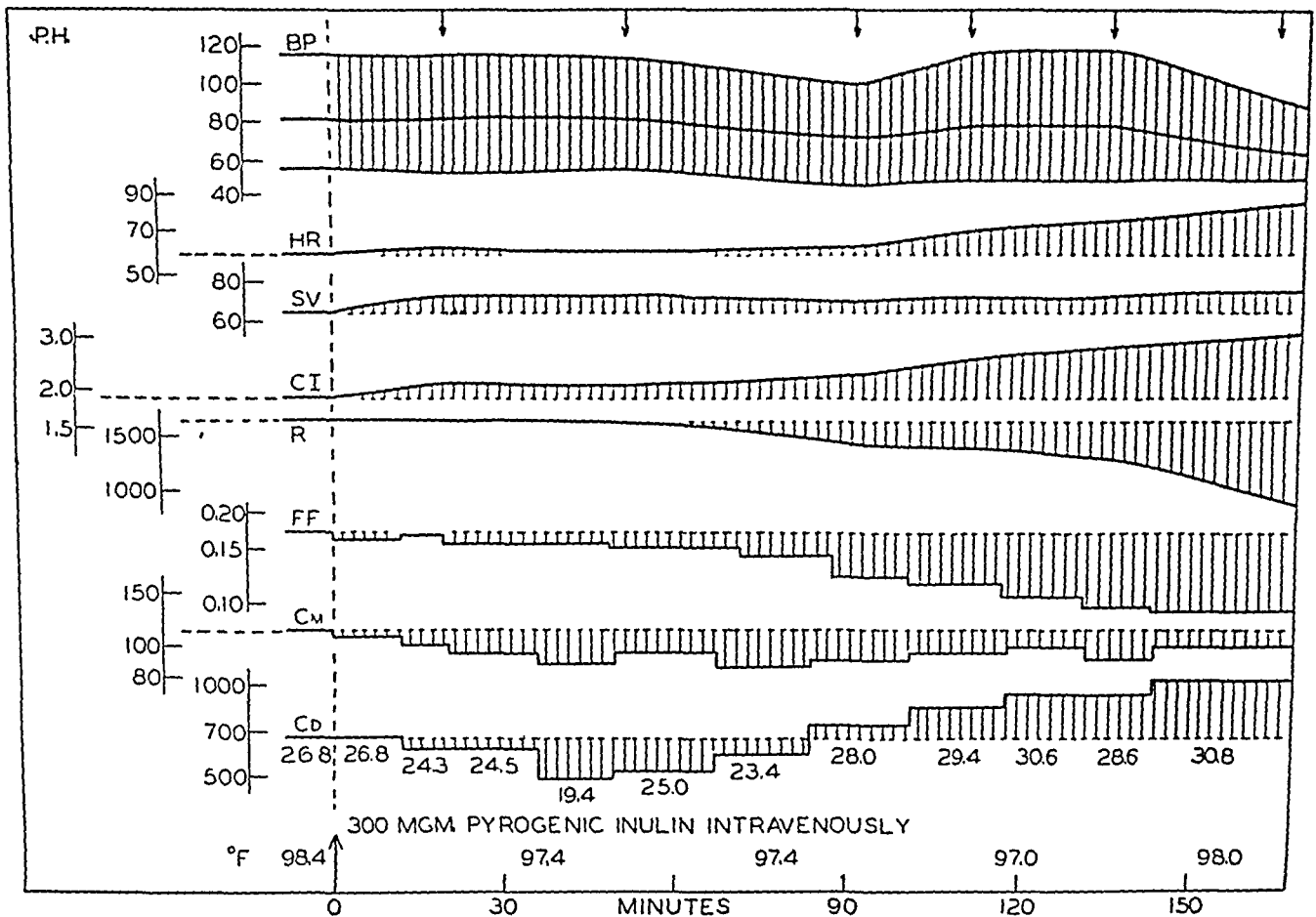


FIG. 1. SUBJECT P. H. THE SYSTEMIC AND RENAL CIRCULATORY EFFECTS OF THE PYROGENIC REACTION, NORMAL, MALE, 52 YEARS

BP, blood pressure (Hamilton manometer) in mm. Hg, the thick line being the mean pressure; HR, heart rate; SV, stroke volume in ml.; CI, cardiac index (ballistocardiograph) in liters per minute per square meter of body surface; R, peripheral resistance in dynes cm^{-5} sec.; FF, filtration fraction (fraction of renal plasma flow filtered at the glomerulus); C_m , mannitol clearance (glomerular filtration rate in ml. per minute); C_D , diodrast clearance (renal plasma flow in ml. per minute). Values of the renal fraction (per cent of the cardiac output passing through the kidneys) are inserted below each diodrast clearance period figure; °F, rectal temperature in degrees of Fahrenheit. The arrows at the top of the figure indicate times at which hemodynamic data were obtained. All subjects described here were premedicated with amidopyrine.

Following the intravenous injection of 300 mgm. of pyrogenic inulin at zero time the cardiac output slowly rose and peripheral resistance decreased. The blood pressure fell from a control value of 116/58 to 90/50, the mean pressure changing very little. The filtration rate decreased and the renal plasma flow, after falling during the first hour following injection of the pyrogen, increased markedly. The filtration fraction fell steadily. The blood pressure was well maintained despite the decrease in peripheral resistance and striking renal vasodilation. This subject remained completely asymptomatic.

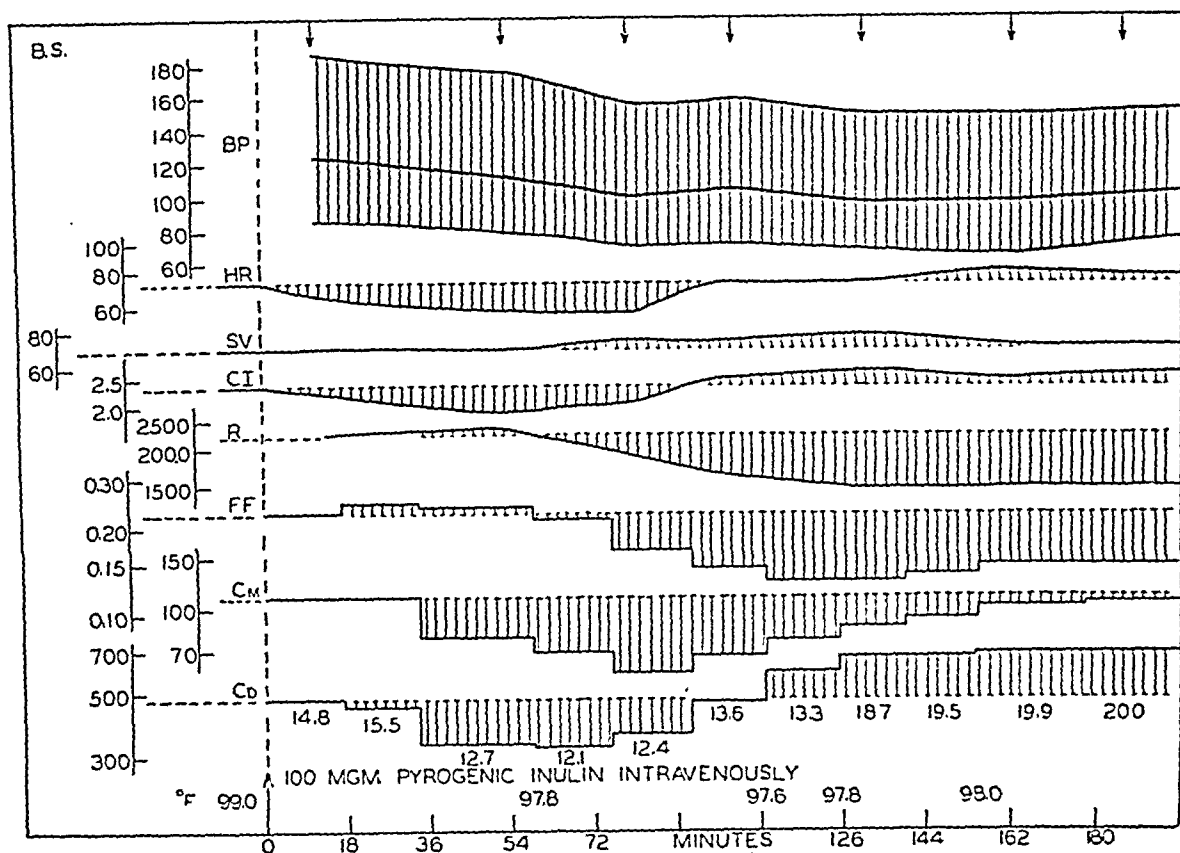


FIG. 2. SUBJECT B. S. (SEE FIGURE 1 FOR SYMBOLS). ESSENTIAL HYPERTENSION, FEMALE, 44 YEARS

Pyrogenic inulin (100 mgm.) was administered at zero time. The blood pressure fell from 185/84 to 150/70, largely as a result of a decreased peripheral resistance. There was little change in cardiac output. The renal events were characteristic, except that a marked decrease in glomerular filtration rate was observed during the first 2 hours.

A second normal subject (S. V.) was given 150 mgm. of pyrogenic inulin. Cardiac output, measured by both the direct Fick method and ballistocardiograph, increased by 54 per cent and 64 per cent, respectively, over a period of 3 hours following administration of pyrogen. Arterial pressure showed no significant change and, though followed for several hours, no trend toward hypotensive levels was noted. The peripheral resistance fell as cardiac output increased. Renal function was not measured in this patient. On return to the ward, he became ambulatory immediately without ill effect.¹

¹ The cardiovascular changes during the afebrile pyrogen reaction were qualitatively similar in these 2 patients regardless of the method employed in determining cardiac output. However, a comparison (S. V.) of the values of cardiac output obtained by the 2 methods revealed a striking discrepancy at the height of the pyrogenic reaction. The control values of cardiac index agreed fairly well.

Since the findings of increased cardiac output, decreased peripheral resistance and fall in blood pressure in these 2 subjects agreed with those of other workers (3, 2, 4) and since these responses were typical of the many pyrogenic reactions we have observed in normal man, it was felt that further control studies were unnecessary.

Hypertensive subjects

In 2 of 6 hypertensive subjects (B. S., M. P.) the response to pyrogen was essentially similar to the direct Fick method giving a figure of 4.22 l. per min. per m², the ballistocardiograph (BCG) 3.94 l. per min. per m². Following the injection of pyrogen the cardiac index was increased to 5.07 l. per min. per m² (BCG) and 6.9 (direct Fick). The discrepancy indicates that caution must be exercised in accepting the latter values of cardiac output determined by the ballistocardiograph during unusually rapid arterial oscillations. The slow rate of change, tends to minimize apparent increases in cardiac output and decreases in peripheral resistance.

that of the normotensives described above. The hemodynamic changes observed in 1 of these (B. S.) are illustrated in Figure 2. A gradual fall in arterial pressure without much change in pulse pressure followed the injection of pyrogenic inulin. Cardiac output, pulse rate and renal plasma flow increased, while a significant and progressive diminution of the peripheral resistance was noted. Blood pressure levels were not measured following completion of the illustrated part of the study; however, the patient remained in bed and asymptomatic. Abnormalities of the ballistocardiographic record made measurement of the cardiac output impossible in subject M. P., a young female with hypertension of at least 2 years'

duration. However, as among the normal subjects, no significant change in arterial pressure was observed, following the administration of typhoid vaccine although the reaction elicited renal hyperemia associated with a decreased filtration fraction. In one respect this subject responded in an abnormal manner. At the peak of the pyrogenic reaction, as revealed by renal hyperemia, it was found that assumption of the upright position resulted in the rapid onset of dizziness and faintness associated with tachycardia. Unfortunately, it was impossible to obtain a record of blood pressure at this time. Return to the horizontal position quickly relieved the symptoms and the subject remained asymptomatic, with an unchanged

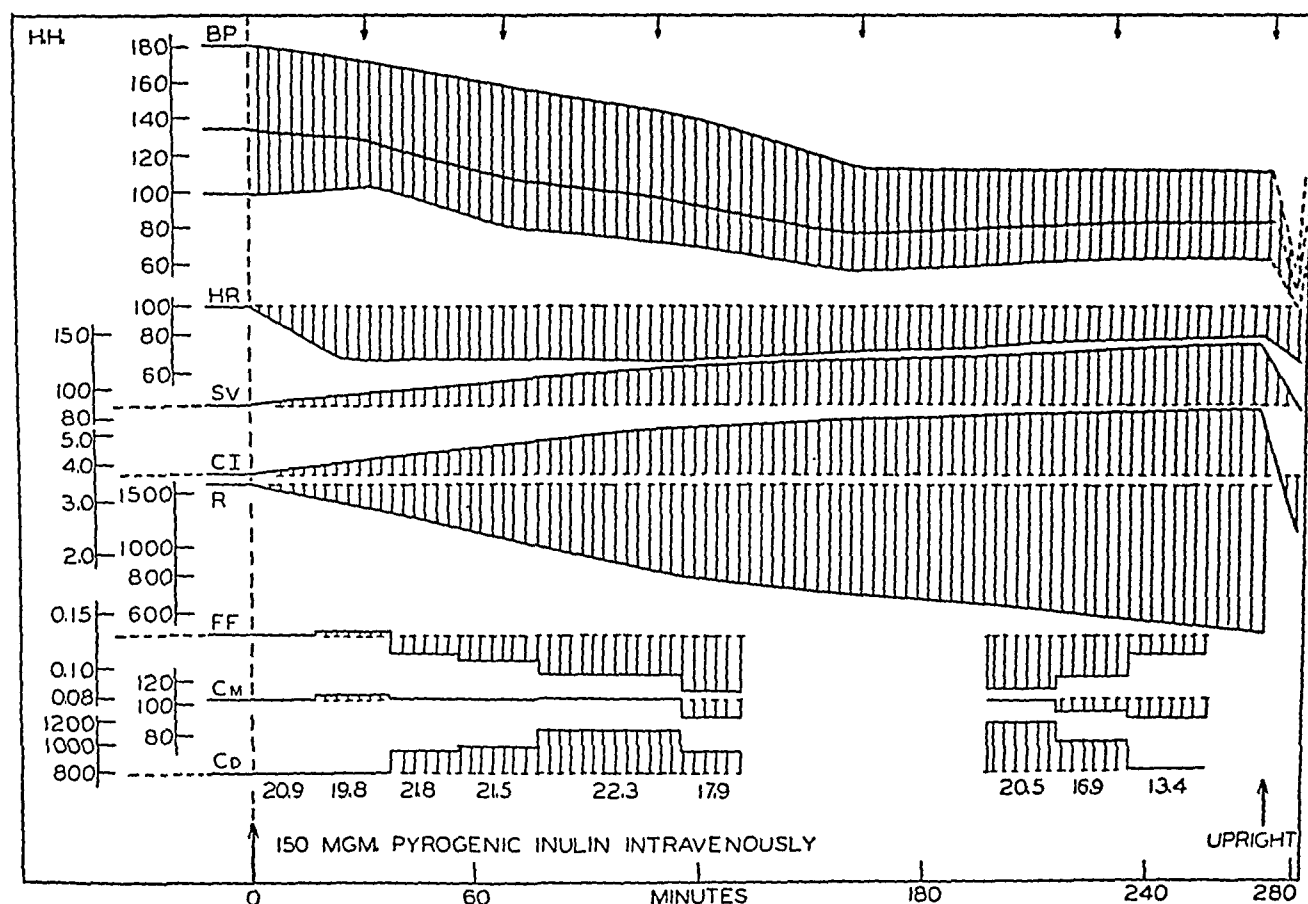


FIG. 3. SUBJECT H. H. (CI, CARDIAC OUTPUT IN LITERS PER MINUTE PER SQUARE METER OF BODY SURFACE MEASURED BY DIRECT FICK METHOD; SYMBOLS OTHERWISE AS FIGURE 1.) ESSENTIAL HYPERTENSION, MALE, 49 YEARS

Following administration of 150 mgm. of pyrogenic inulin the cardiac output steadily increased as a result of an increased stroke volume, while the peripheral resistance fell. Blood pressure fell over a period of 3 hours to normotensive levels where it was well maintained by reciprocal changes in cardiac output and peripheral resistance. Renal plasma flow increased during the first 4 hours and then returned to the control level. There was very little change in the filtration rate and the filtration fraction decreased. At upright, the subject was placed in a sitting position and hypotension (interrupted pressure curve indicates sphygmomanometric measurements) developed associated with a marked fall in cardiac output. Recovery followed the return to recumbency. Body temperature, not recorded here, remained constant throughout.

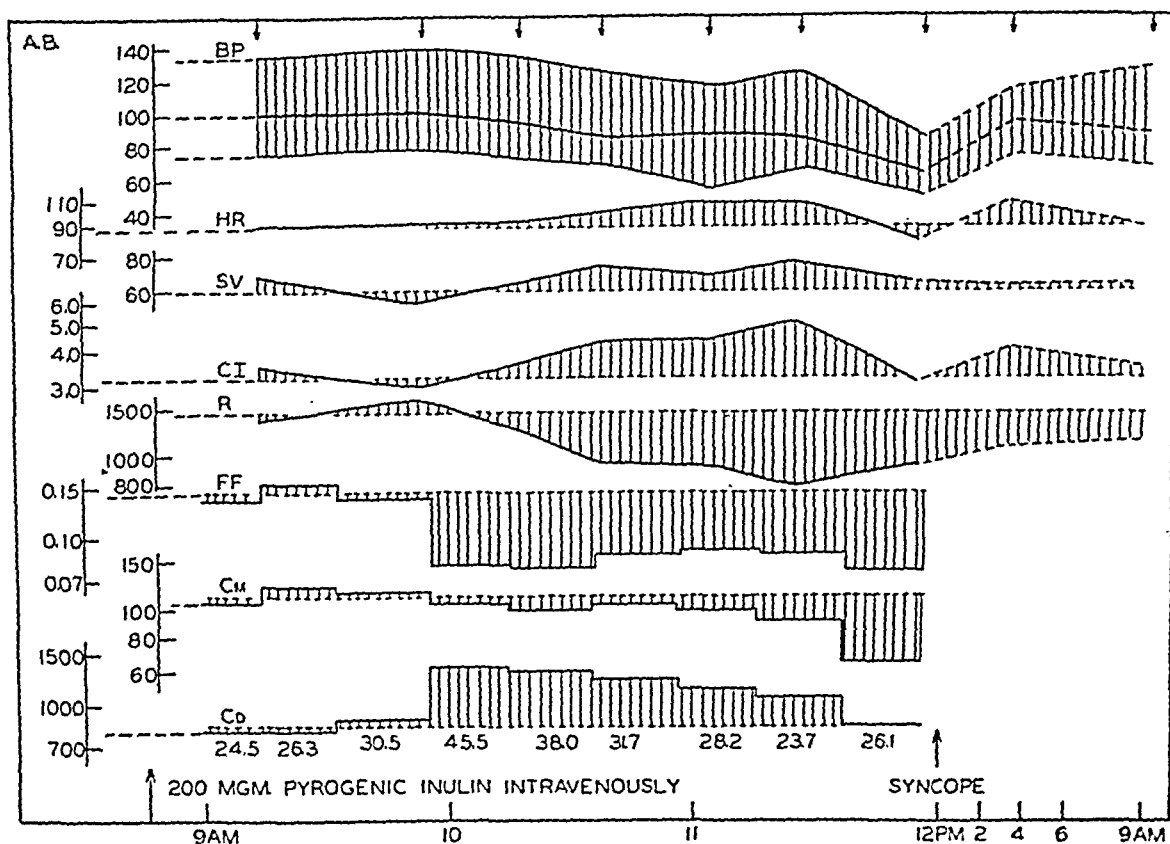


FIG. 4. SUBJECT A. B. ESSENTIAL HYPERTENSION, MALE, 33. (SEE FIGURE 1 FOR SYMBOLS)

The blood pressure fell very little during the first 3 hours following the administration of 200 mgm. of pyrogenic inulin. The decrease in peripheral resistance during this period was balanced by an increase in cardiac output. At 12:00 p.m., the renal function studies were completed and the patient rose to his feet. Very shortly thereafter syncope occurred and blood pressure could not be obtained. Interrupted pressure curve marks sphygmomanometric measurements. It should be noted that cardiac output and pulse rate decreased just prior to the onset of syncope. Body temperature, not recorded here, was normal throughout.

blood pressure as long as she did not sit or stand up. This abnormal response to postural change cleared slowly during the following 12 hours.

In the remaining subjects (H. H., A. B., M. N., G. O.) the evidence of maladjustment to postural change was clearly demonstrated. In every case the assumption of the standing or sitting position was followed by hypotension and faintness or syncope several hours after injection of pyrogen. In 1 (G. O.) impending syncope was present in the horizontal position. These subjects will be described in order of the severity of the hypotensive response.

Subject H. H. (Figure 3) showed a typical response. The cardiac output (direct Fick) increased markedly, the peripheral resistance decreased and the blood pressure fell slowly to a normal level. In the late afternoon, after the

termination of the renal studies and more than 4 hours after pyrogen had been administered, he was raised to a sitting position. At this time the blood pressure had fallen to normotensive levels, cardiac output had increased and the peripheral resistance had fallen to a markedly low level. On sitting up, the blood pressure (measured sphygmomanometrically) fell almost immediately to a level of 60/40 and bradycardia appeared. A direct Fick determination was made immediately (in recumbency) and the cardiac output was found to have fallen well below the control value. On completion of the Fick measurement, arterial pressure as determined by the Hamilton manometer had returned to the level existing before the subject was elevated. It was found that the pressure fell rapidly each time he was brought to the sitting position, with rapid restoration on return.

ing the supine posture. No attempt was made to calculate the peripheral resistance during these tests because of the difficulty of measuring cardiac output by the direct Fick method and determining the arterial pressure simultaneously.

Subject A. B. (Figure 4) was a 33-year-old male with essential hypertension of at least 2 years' duration. His blood pressure fluctuated widely, falling to normal levels on bed rest. Following administration of pyrogen, the arterial pressure fell slowly with a narrowing of the pulse pressure. As renal hyperemia appeared the cardiac output, stroke volume, and pulse rate increased with a significant diminution of peripheral resistance. At the termination of the renal function study a marked drop in the blood pressure associated with a fall in cardiac output was noted. A few minutes later, on rising to his feet, the

subject suddenly became pulseless and fainted. Several hours later the blood pressure was found somewhat higher than just prior to syncope and the cardiac output was again above control values. The peripheral resistance continued to be depressed. The following morning the blood pressure and cardiac output had returned to the control level.

Figure 5 illustrates a similar reaction observed in another young hypertensive, M. N. She was examined again 3 hours after the termination of the renal function study and by this time was found to have a markedly reduced arterial pressure and pulse pressure associated with a falling cardiac output. Soon after she was placed in a wheel chair to return to the ward, she suddenly became pulseless and complained of faintness. This marked hypotensive state lasted for several

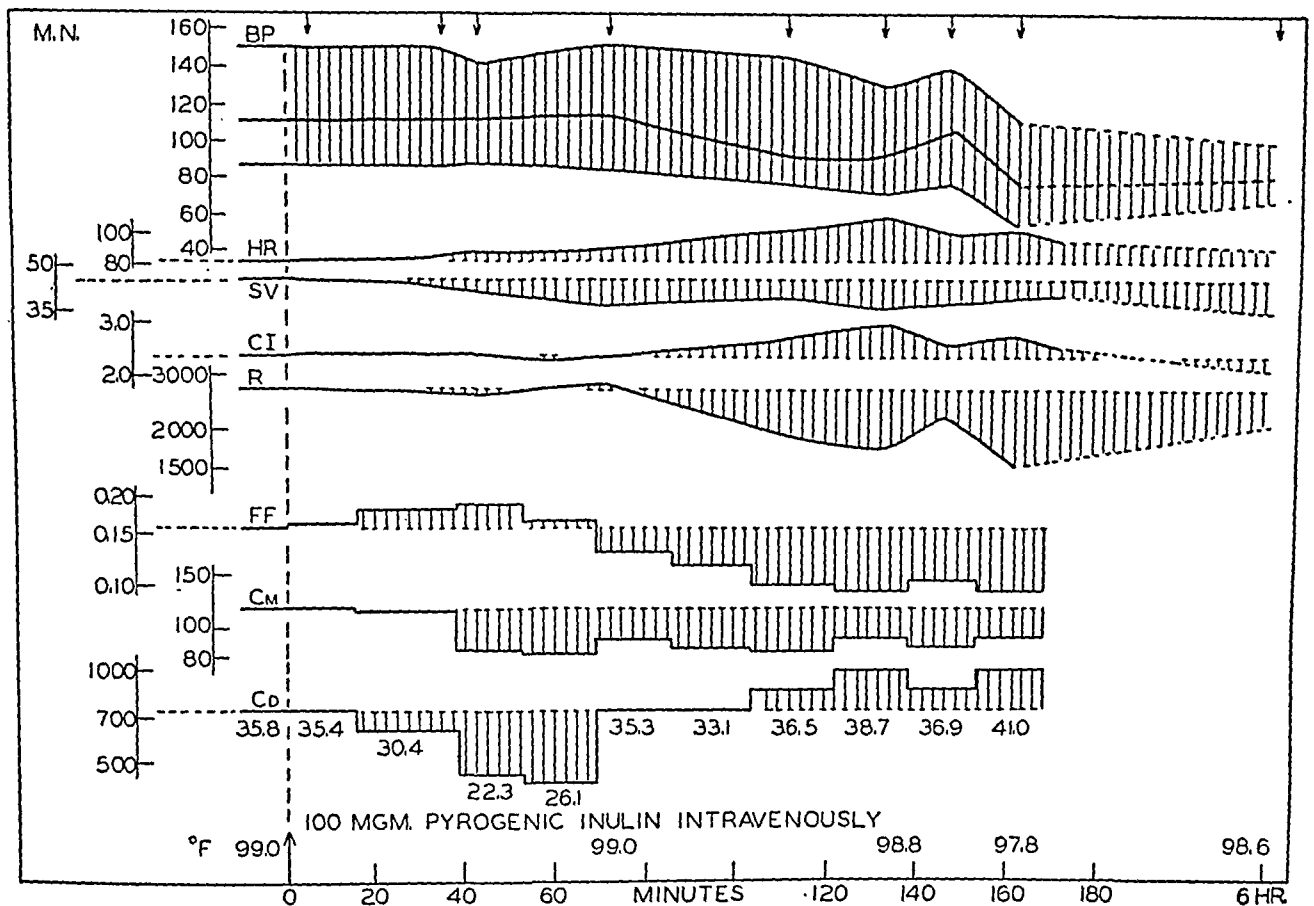


FIG. 5. SUBJECT M. N. ESSENTIAL HYPERTENSION, FEMALE, 20. (SEE FIGURE 1 FOR SYMBOLS)

The arterial pressure changed very little during the first 2 hours after the administration of 100 mgm. of pyrogenic inulin. Peripheral resistance fell while cardiac output increased. Six hours after the pyrogen was administered it was found that the arterial pressure (interrupted pressure curves denote sphygmomanometric measurements) had fallen to hypotensive levels, associated with a decrease in cardiac output below the control value. The peripheral resistance remained reduced. Shortly after this measurement was made, the subject became pale, pulseless and dizzy. She remained so for several hours.

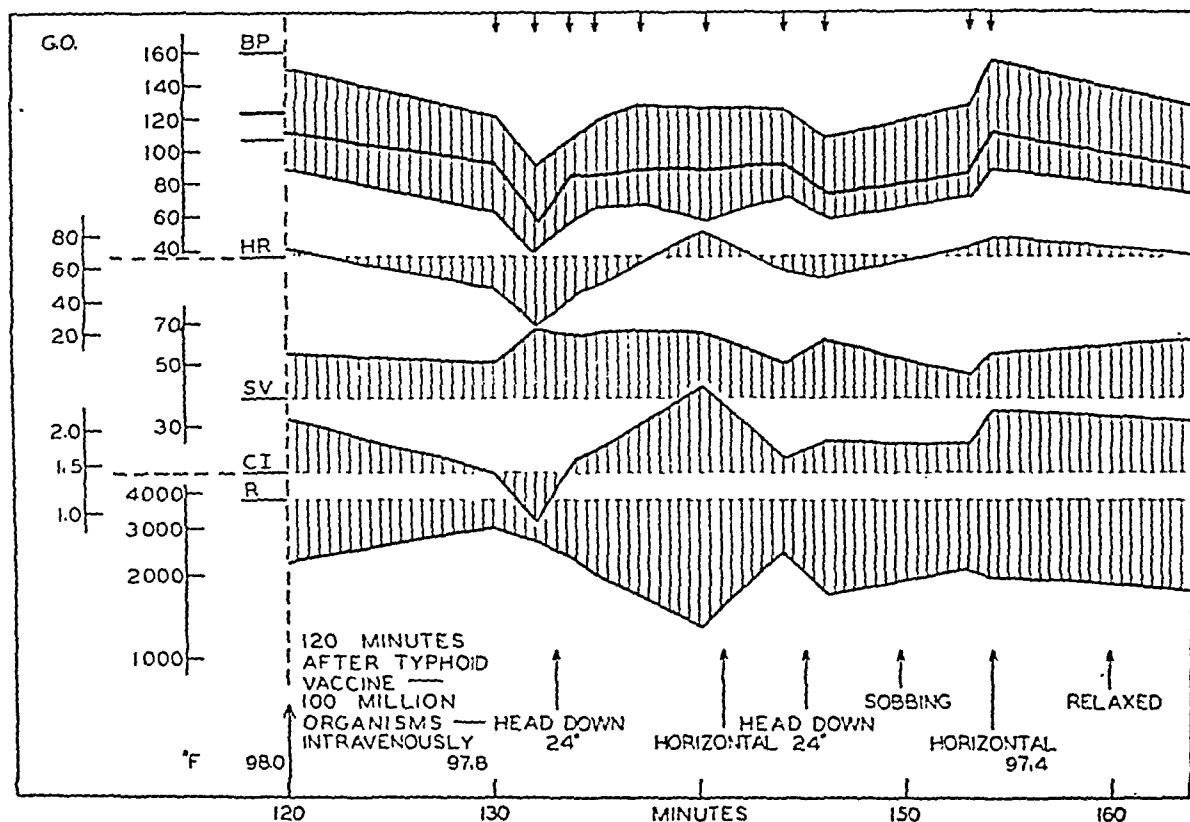


FIG. 6. SUBJECT G. O. ESSENTIAL HYPERTENSION, FEMALE, 44. (SYMBOLS SAME AS FIGURE 1)

No hemodynamic change other than a falling peripheral resistance and an increasing cardiac output was noted during the first 2 hours after pyrogen (typhoid vaccine) was administered. During the next 10 minutes, however, the blood pressure fell to a normotensive level and then fell precipitously to 92/40 mm. Hg associated with a parallel decrease in cardiac output. The peripheral resistance increased slightly. Restoration to a normotensive level followed a change of position from recumbency to 24° head down. The recovery was associated with an increased cardiac output and a further fall in peripheral resistance. The return to the horizontal position brought about a similar sequence of events and the head down position was necessary to avert syncope and relieve faintness. At 150 minutes the patient was questioned about a relative whom she was known to dislike, and she broke into tears and sobbing and shortly thereafter it was found that the blood pressure had returned to a hypertensive level. She was again placed in the horizontal position and the pressure slowly returned to normotensive levels where it remained for approximately 24 hours.

hours associated with faintness, nausea and vomiting, marked pallor and cold extremities.

Subject G. O. (Figure 6) was a 44-year-old female with a well-established hypertension of 3 years' known duration. For 2 hours following administration of pyrogen the blood pressure was well sustained and cardiac output increased while peripheral resistance slowly fell. Then, while still in the horizontal position, a drop in blood pressure to normotensive levels occurred followed by a further rapid fall of systolic pressure to 90 mm. Hg and diastolic to 40 mm. Hg, associated with marked bradycardia, faintness, and nausea. Although peripheral resistance increased somewhat

during the pressure fall, the most striking hemodynamic change was the marked fall in cardiac output. When the subject was placed in the shock position, the blood pressure returned to normotensive levels, the cardiac output increased as a result of an increased heart rate and a larger stroke volume, with subjective improvement. On the return to the horizontal, circulatory changes similar to those seen during the initial episode rapidly recurred. Again the shock position resulted in improvement. At this time the subject was questioned concerning a member of her family against whom she was known to harbor a strong grievance and a stormy emotional response was

elicited. The blood pressure rose quickly to a hypertensive level apparently as a result of an increase in cardiac output due to an increased heart rate and increased stroke volume, the peripheral resistance showing little change. Following the return to horizontal the pressure slowly returned to a normotensive level where it remained for approximately 18 hours. During most of this time the subject was maintained in shock position to assure maximal comfort.

Renal fraction

The percentage of the cardiac output diverted to the kidneys (renal fraction) was calculated in every instance in which simultaneous measurement of renal blood flow and cardiac output was made. The figures are inserted below the graphic record of the diodrast clearances in Figures 1 to 5. The renal fraction increased in every instance over the control figure, usually reaching a maximum at the peak of renal hyperemia. On 3 (P. H., B. S., M. N.) of the 5 occasions in which the renal fraction was measured it fell simultaneously with the renal blood flow shortly before the appearance of renal hyperemia. This phase of decreased renal fraction and renal blood flow was usually associated with a reduction in the filtration rate without much change in the filtration fraction. These phenomena appear to be independent of hypertensive disease and, though not consistently present, appear frequently enough to be noteworthy (2).

DISCUSSION

It is clear from this study that peripheral vasodilation is the primary hemodynamic change in both normal and hypertensive subjects during the pyrogenic reaction. Qualitatively the 2 groups behaved similarly, but there appear to be quantitative differences in the hemodynamic alteration and in the extent to which adjustment is possible. In normal subjects and in some hypertensives, augmentation of cardiac output compensates for the decrease in peripheral resistance and arterial pressure is maintained. But in some hypertensive subjects (H. H. at the beginning and G. O. at the close of the study) the arterial pressure fell to normotensive levels and was thereafter maintained constant. In 1 hypertensive subject (G. O.), ad-

justment failed completely and the blood pressure fell to an alarmingly low level. In every instance the fall in blood pressure could be attributed to the failure of cardiac output to increase in proportion to the dilatation of the peripheral vascular bed.

Hypertensive subjects, as a group, appear to be particularly vulnerable to conditions demanding excessive circulatory adjustments. It is well known (15) that orthostasis is associated with reflex vascular changes by which return of blood to the heart is sustained in spite of gravitational pooling of blood in the extremities. It is presumed that the essential mechanisms involved in these adjustments are much the same as in phases of circulatory inadequacy induced by pyrogen. During the pyrogenic reaction in normal subjects, these adjustments appeared to be adequate (P. H., S. V.) but among hypertensive subjects orthostasis was not efficiently compensated, cardiac output decreased excessively and, as a result, hypotension and syncope ensued. Improvement of return of blood to the heart, by placing the subject in the shock position or by the maneuvers of sobbing, as in patient G. O., resulted in prompt alleviation of symptoms and increase in intra-arterial pressure. It should be noted, however, that the arterial pressure never returned to the control, hypertensive levels. Marked hypotension apparently never followed further decrease in peripheral resistance, but was always attributable to a fall in cardiac output superimposed upon an already dilated vascular bed. As a matter of fact, in G. O. (Figure 6) a compensatory increase in the peripheral vascular resistance was apparent at a time when arterial pressure was falling precipitously.²

Consequently, it seems logical to assign the cause of the hypotension that occurs in hypertensive subjects during the pyrogenic reaction, whatever the precipitating factor, to a disorder of the mechanisms that effect compensatory increases in cardiac output. The increases in cardiac output

² This change in calculated resistance may have resulted from inaccuracies inherent in the ballistocardiographic method of determining cardiac output, for an erroneously low value of cardiac output would produce such an apparent elevation of calculated resistance. The behavior of the peripheral resistance in this situation requires further study with a more precise method for measuring cardiac output.

demanding to maintain pressure levels in our subjects were by no means in excess of daily requirements (16) and in none of these subjects was there any evidence of diminished cardiac reserve. It is likely, therefore, that cardiac output would have been efficiently sustained had an adequate return and filling mechanism been operative. This view is strengthened by the favorable response to measures that improved cardiac filling. In the hypotension of spinal anesthesia it is probable that relaxation of the muscles of the lower extremities interferes with activity of the "booster pump" system of the intramuscular channels and so contributes to a reduction in cardiac output (17), but this factor cannot be evoked to explain the defective return of blood to the heart during the pyrogenic reaction. Moreover, no change in respiratory activity was apparent as a possible cause of an altered gradient of venous pressure between the thorax and the abdomen. It seems more likely that capillary and venous dilatation and inadequate adjustment to postural change were at fault in the studies reported here. Such a dilatation might be expected to pool a large quantity of blood in the lower extremities and abdominal cavity in the upright position or even in recumbency. It is noteworthy that these mechanisms appear to be altered by hypertensive disease, usually considered pre-eminently a disorder of the arterial and arteriolar channels of the circulatory system.

It is probable that the fall in peripheral resistance was a result of arteriolar dilatation, but whether this vasodilation is generalized or confined to particular portions of the vascular bed is undetermined. No change in the blood flow to the skin or skeletal muscle has been noted in normal man during the afebrile pyrogen reaction (18), while it is known that under these conditions the renal blood flow is increased (2) and it is here demonstrated that this increase represents an actual increase in the fraction of the total blood put out by the heart, demonstrating that renal vasodilatation was proportionately greater than dilatation elsewhere in the vascular bed. Hence, the kidney appears to be peculiarly susceptible to the action of pyrogen in both normal and hypertensive individuals.

It has been noted, and confirmed here, that the renal blood flow frequently decreases shortly after

the administration of pyrogen (2). This phenomenon occurs at the time when the chill usually develops in the unpremedicated subject. These workers (3) noted an increase of arterial pressure in unpremedicated subjects during the chill and considered this as indicative of an overall vasoconstriction. However, no vasoconstriction was evident in the values for peripheral resistance or in the arterial pressure in the absence of the chill and fever either in previous studies (2) or in the present series. Nevertheless, the decrease in renal blood flow so often associated with a fall in renal fraction during the early or latent period denotes the presence of vasoconstriction in the kidney, a phenomenon which presumably is associated with vasodilatation elsewhere since it is not reflected in any change of total peripheral resistance or mean arterial pressure. The identity of the opposing dilated vascular beds remains obscure. The failure of the filtration fraction to show any consistent change during the vasoconstrictor phase is evidence that both afferent and efferent glomerular arterioles contribute to the reduction in size of the renal vascular bed.

SUMMARY

1. A study of the hemodynamic changes induced by the afebrile pyrogenic reaction (in premedicated subjects) has been made in 2 normal and 6 hypertensive persons.

2. In every instance cardiac output increased as a result of an increase in both pulse rate and stroke volume, while total peripheral resistance decreased.

3. In normal subjects and in some hypertensive individuals reciprocal changes in peripheral resistance and cardiac output resulted in adequate maintenance of arterial pressures. In 2 hypertensive subjects, however, arterial pressure fell to normotensive levels and in a third, marked hypotension developed. In the latter, a change to head-down position was necessary to maintain the blood pressure at physiologically adequate levels.

4. Normotensive subjects are capable of adequate vascular adjustment to postural change during the pyrogenic reaction. Among hypertensives, however, even though adjustment may be adequate in the recumbent position, orthostasis may

result in a shock-like state. The observations reported here indicate that a fall in cardiac output is responsible for circulatory inadequacy under these conditions.

5. Renal hyperemia occurred in each instance studied and in the 5 subjects in whom cardiac output and renal blood flow were measured simultaneously, the renal fraction increased, implying relatively greater vasodilation in the renal vascular bed than in the rest of the circulatory system.

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THE EFFECT OF SALICYLATES ON THE ELECTROLYTE STRUCTURE OF THE BLOOD PLASMA.¹ I. RESPIRATORY ALKALOSIS IN MONKEYS AND DOGS AFTER SODIUM AND METHYL SALICYLATE; THE INFLUENCE OF HYPNOTIC DRUGS AND OF SODIUM BICARBONATE ON SALICYLATE POISONING

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The clinical picture typical of salicylate poisoning has often been described: hyperpnea appears to be the most prominent symptom; other symptoms are vomiting, thirst, sweating, diuresis, fever, and various signs of irritation of the central nervous system, including convulsions followed by depression and coma. Albumen, casts, and red and white cells are often found in the urine. The physiologic mechanism underlying the hyperpnea of salicylism has been the subject of much discussion. As the dyspnea of salicylate poisoning was noted to resemble the Kussmaul type of breathing of diabetic coma, ketone bodies were suggested as a common cause for the acidosis and hyperventilation in both conditions (1). This hypothesis was not borne out in later studies, which did not reveal increased ketonemia in man or rabbits receiving large doses of salicylates (2). In more recent times the theory of a primary metabolic acidosis, accepted by many clinical observers, appeared to be supported by the finding of a decreased bicarbonate content of the blood, but the origin of this presumed acidosis has remained obscure (3 to 5). Increased amounts of lactate found in the blood of rabbits following sodium salicylate administration were thought to be partly responsible for the presumed acidosis (6). The significance of this observation appears uncertain, since the increases varied widely, and the muscular effort involved in hyperventilation may have accounted for them. A renal mechanism for the metabolic acidosis has been suggested (7, 8) but the rapidity of the onset of the hyperpnea and the inconstancy and mildness of the renal changes appear difficult to reconcile with such an hypothesis. Another group of observers has favored the

view that the hyperpnea is of central origin and that changes in the blood are secondary. In a study of salicylate poisoning in man the finding of insignificant changes in the blood pH in the presence of lowered concentrations of bicarbonate suggested that the hyperpnea must result from central stimulation by the drug (9). Others (10, 11) came to the same conclusion on the basis of short-time experiments with small doses of sodium salicylate in man, in which lowered CO₂ tensions and alkaline urines were observed coincidentally with unchanged values for the alkali reserve of the blood. A critical evaluation of the literature led to the conclusion that the cause of the hyperpnea was both central and metabolic (12). In a recent study dealing experimentally with the effects of toxic doses on the composition of the blood of dogs (13), a pH of 7.61 concomitant with a CO₂ content of 13 m.eq. per liter was reported in 1 dog and mention was made of unpublished experiments, which also indicated the presence of at least transient alkalosis following the administration of salicylates. In this study, changes in the heat regulation were stressed as an explanation for the hyperpnea of salicylism. In all, it would appear that the data available at present are insufficient to permit a satisfactory description of the electrolyte structure of the blood in salicylate poisoning.

The observation of a case of aspirin poisoning in a 3-year-old child, with the plasma pH elevated and the CO₂ content and tension decreased, led us to investigate the effects of salicylates in monkeys and dogs. Sodium and methyl salicylate were administered to the animals by parenteral routes and blood analyses were performed for pH, CO₂, chloride, sodium, and potassium. The serum protein and the distribution of the acid-soluble phosphorus in the blood of several ani-

¹ A preliminary report was presented before the American Pediatric Society, May 1, 1942 (*Am. J. Dis. Child.*, 1942, 64, 201).

mals were determined. The effects of several hypnotic drugs and of sodium bicarbonate on the hyperpnea of salicylism were also studied.

METHODS

Rhesus monkeys weighing 3 to 5 kgm., and male mongrel dogs weighing 14 to 20 kgm. were used. The animals were fasted 24 hours before and during the experimental periods but were allowed free access to water at all times. Weight, temperature, and respiratory rate were recorded in all, and urinary output in some of the dogs. The dogs lost on the average 1.5 kgm. of weight during the experiments. The respirations of all animals increased greatly in frequency and depth following the administration of salicylates. Two types of hyperventilation were observed occurring in the same animals alternately within short periods of time: (1) deep labored breathing reaching frequencies as high as 150 per minute; and (2) panting, much more shallow in character, at rates of 250 or more respirations per minute. During the later stages of fatal intoxication or under the influence of hypnotic drugs, the first type of breathing predominated and panting was observed only occasionally. The temperature of the animals rose in a few instances to values as high as 41° C., but often remained at 39° C. The urinary output was greatly increased in the beginning of the intoxication, and diminished during the later stages.

Blood samples from the femoral artery were drawn with a tightly-fitting syringe and needle and were delivered under paraffin oil. The serum pH values were determined with a glass electrode at 38° C. with precautions against exposure to air. Serum CO₂, chloride, phos-

phorus, and protein values were determined by methods previously described (14), sodium by the method of Leva (15), and potassium according to the slightly-modified method of Consolazio and Talbott (16). The CO₂ tension was calculated from the values for pH and bicarbonate. Data on the hemoglobin, the red count, and the packed volume of red cells of several animals did not reveal any significant change, and, therefore, are not reported here.

EXPERIMENTATION WITH SODIUM SALICYLATE

In Table I are shown representative experiments on the effect of sodium salicylate on monkeys and dogs:

Rhesus monkey number 400, weighing 3.6 kgm., received repeated intravenous injections of sodium salicylate. The animal presented signs of poisoning closely resembling those seen in man, including hyperpnea, flushing of the face, weakness, and profuse diuresis. The chemical data on the blood taken 1 hour after the last dose of sodium salicylate, when the animal appeared very weak, showed an extraordinarily elevated pH, a moderately reduced CO₂ content, and a greatly decreased tension of CO₂.

Dog S1 received a total of 1.0 gram per kgm. of sodium salicylate intraperitoneally in divided doses over a period of 24 hours. Extreme hyperpnea developed shortly after the first dose of 0.2 gram per kgm. had been administered and continued until the dog died. Diuresis, great weakness, and hyper-reflexia were noticed among the signs of intoxication.

The dogs 839 and 840 were studied with the purpose of following the effect of single intravenous doses of sodium salicylate on the electrolyte equilibrium. Dog 840

TABLE I
Effect of sodium salicylate on the ionic equilibrium of blood plasma

Animal	Number	pH	CO ₂ <i>m. eq. per liter</i>	pCO ₂ <i>mm. Hg</i>	Chloride <i>m. eq. per liter</i>	Comment
Monkey	400	7.85	16.5	9	97.3	Received 1.1 grams per kgm. intravenously in 5 divided doses over a period of 30 hours. Sample 1 hour after last dose. Panting, flushed face, weak. Sacrificed.
Dog	S1	7.44	25.7	38	102.2	Preliminary sample, followed by intraperitoneal injections of 0.2 gram doses of sodium salicylate. Wt. 14 kgm.
		7.49	24.2	32	105.3	4 hours later; total dose 0.4 gram per kgm.; breathing fast.
		7.66	23.1	20	110.9	24 hours later; total dose 1.0 gram per kgm.; panting hard, weak. Died 1 hour after sample.
Dog	840	7.42	20.5	31	112.9	Preliminary sample, followed by intravenous injection of sodium salicylate, 0.6 gram per kgm. Weight 17 kgm.
		7.62	17.9	17	111.3	45 minutes later; panting hard.
		7.50	19.2	24	108.4	3 hours later; very weak; died $\frac{1}{2}$ hour later.
Dog	839	7.48	22.3	30	113.4	Preliminary sample, followed by intravenous injection of sodium salicylate, 0.3 gram per kgm. Weight 15 kgm.
		7.49	22.2	29	109.2	20 minutes later; breathing rapidly.
		7.58	22.5	24	109.6	1.5 hours later; panting.
		7.54	22.9	27	107.2	5 hours later; hyperpnea diminished.
		7.43	22.0	33	103.9	23 hours later; appears normal.

TABLE II
Effects of methyl salicylate on plasma electrolytes

Dog	pH	CO ₂ <i>m. eq. per liter</i>	pCO ₂ <i>mm. Hg</i>	Chloride <i>m. eq. per liter</i>	Sodium <i>m. eq. per liter</i>	Comment
Pr.	7.42	25.4	40	111.4	150.3	Preliminary sample, followed by intramuscular injection of 2 ml. doses of methyl salicylate. Weight 15 kgm.
	7.41	18.6	28	106.6		30 hours later; total dose 14 ml.
	7.41	12.9	19	102.6	129.6	53 hours later; total dose 18 ml.; panting, weak; died 8 hours later.
Pa.	7.48	22.7	32	118.1	148.7	Preliminary sample, followed by intramuscular injection of 2 ml. doses of methyl salicylate. Weight 31 kgm.
	7.49	13.7	17	113.4	141.8	44 hours later; total dose 16 ml.; hyperpnea intense.
	7.49	12.8	16	106.9	137.5	68 hours later; total dose 23 ml.; seems very weak, died $\frac{1}{2}$ hour later.

received what turned out to be a lethal dose, 0.6 gram per kgm. Within 20 minutes of the injection, the animal was breathing very deeply and continued to do so until its death 3½ hours later. The sample taken 45 minutes after the injection showed marked elevation of the pH, a slight drop in the content of CO₂, and a marked drop in the tension of the CO₂. The 3-hour sample showed less deviation from the normal in values for pH and CO₂, but the animal at that time appeared very weak and was breathing less forcefully. Dog 839 received only 0.3 gram per kgm. of sodium salicylate and tolerated the drug well. The rise in pH and fall in CO₂ tension were at their height 1½ hours after the injection. After 5 hours, hyperventilation was present to a moderate extent, and when the last blood sample was taken 23 hours after the injection, the respiration had returned to normal. The CO₂ content of the serum changed little during the experiment.

The serum chloride of these animals did not exhibit marked deviations from the normal.

THE EFFECT OF METHYL SALICYLATE

Methyl salicylate was substituted for sodium salicylate in the later experiments in order to avoid the complicating effect of sodium ions. The drug was always administered intramuscularly.

Table II presents data on the changes of the serum electrolytes in 2 dogs that received repeated injections of methyl salicylate totaling 18 and 23 ml. respectively. The serum pH of these animals changed little, but both CO₂ content and CO₂ tension of the serum were markedly lowered. The chloride and the sodium levels also decreased. In Figure 1 are presented data on a third animal which received repeated injections of the drug in increasing amounts over a period of 4 days. The serum pH of this dog reached a peak of 7.64 and when the animal developed what were thought to be tetanic convulsions dropped rather sharply. The content and tension of CO₂ in the serum of this dog decreased to levels of 10.7 m. eq. per liter, and 12 mm. Hg, respectively, the sodium to a level of 124 m. eq. per liter and with it the chloride (not shown) to 94 m. eq. per liter. The effect of a single

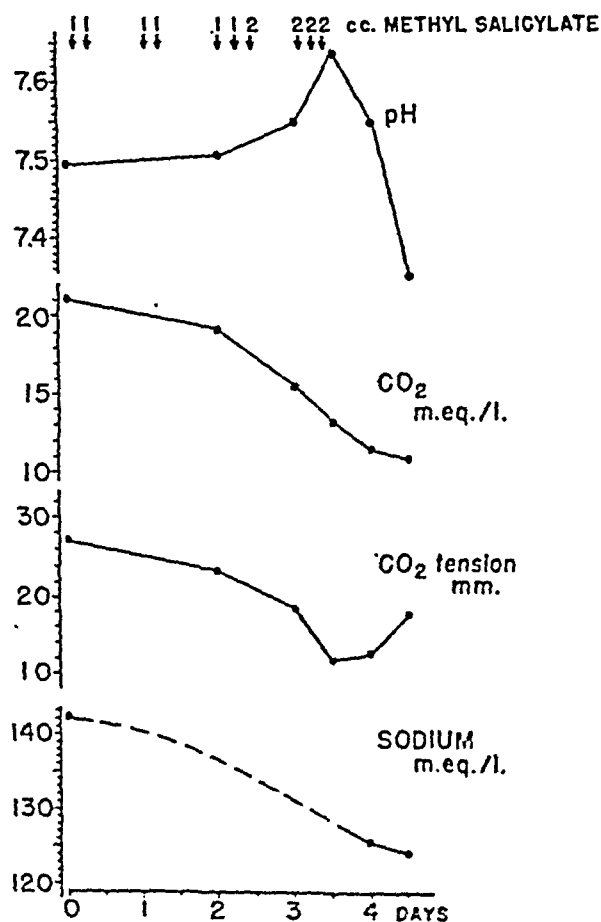


FIG. 1. THE EFFECT OF REPEATED DOSES OF METHYL SALICYLATE

Dog weighing 14 kgm. received intramuscularly the amounts of methyl salicylate indicated in the figure. The animal began to hyperventilate air only on the third day. On the fourth day, the animal was weak and labored, hyper-reflexia and muscular contractions. It was moribund when the last blood sample was taken.

large non-fatal dose of methyl salicylate on pH and CO_2 of the serum of another dog, who received 10 ml. of the drug, is shown in Figure 2. The greatest change took place after about 24 hours, with a slow return to normal values over a period of 4 days.

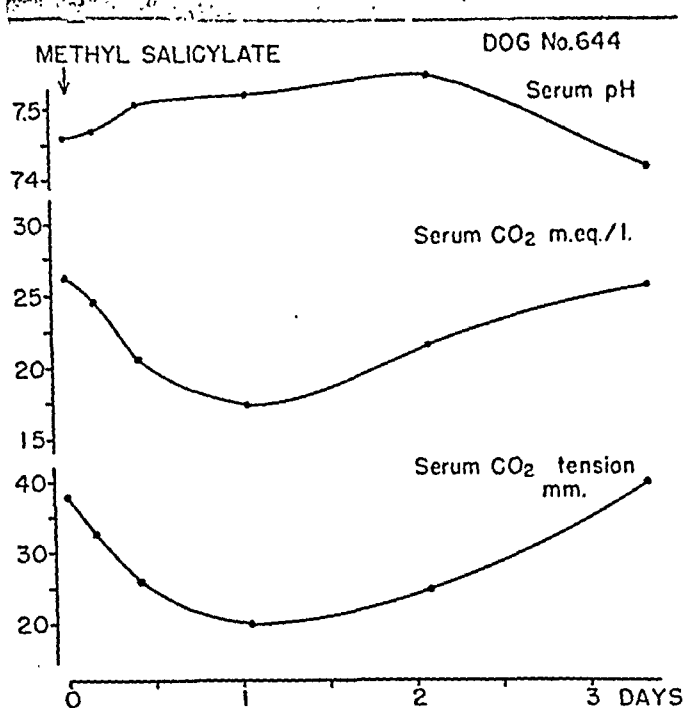


FIG. 2. THE EFFECT OF A SINGLE DOSE OF METHYL SALICYLATE

Dog 644, weighing 15 kgm., received 10 ml. of methyl salicylate intramuscularly. Within a few hours the respirations increased, to reach a maximum about 24 hours after the injection, and decreased after 48 hours.

In Table III are presented data on the sodium, potassium, and chloride content of the sera of 9 dogs all of whom developed the typical signs and chemical changes of salicylism, after having received 10 ml. of methyl salicylate. The sodium was decreased in 5, and little changed

TABLE III

Sodium, chloride, and potassium in the plasma of dogs 24 hours following methyl salicylate administration

Dog number	Before			After		
	Sodium	Chloride	Potassium	Sodium	Chloride	Potassium
	<i>m. eq. per liter</i>	<i>m. eq. per liter</i>	<i>m. eq. per liter</i>	<i>m. eq. per liter</i>	<i>m. eq. per liter</i>	<i>m. eq. per liter</i>
645	150	117	6.2	143	112	3.4
932	141	104	5.0	134	103	4.6
933	145	108	4.2	128	96	2.7
937	149	119	6.8	134	108	2.9
938	148	117	5.1	142	115	4.0
936	141	108	4.5	139	101	2.6
940	139	110	4.0	144	119	3.0
941	146	112	4.4	145	110	2.9
Sp.	142	110		146	112	

in the sera of 3 of the animals. In 3 animals both serum chloride and sodium were diminished significantly; in 2 the sodium alone, and in 1 the chloride alone was decreased; the concentration of potassium in the serum fell in every instance.

The distribution of the acid-soluble phosphorus in the blood was determined in several animals. The inorganic phosphorus was regularly decreased, but little change was found in the organic acid-soluble phosphorus content of the erythrocytes. The serum proteins were not significantly altered.

RESULTS

The results of the foregoing experiments may be summarized briefly as follows. In all experiments, reduced tensions of CO_2 in the serum were found. Significant elevation of the serum pH occurred in most instances, but occasionally change of pH was absent. The serum bicarbonate concentration was lowered in all experiments of longer duration, but such change was absent in the animals receiving sodium salicylate intravenously. The serum sodium concentration was lowered in the majority, and the chloride in about half of the dogs. Potassium values were regularly decreased.

The evidence would appear to indicate that salicylates cause primary hyperventilation. The resultant lowering of the CO_2 tension leads to an alkalotic tendency in the blood. The lowering of the bicarbonate concentration may be interpreted as a secondary compensatory change tending to maintain the usual ratio between carbonic acid and bicarbonate, and resulting in a smaller shift of pH than would otherwise occur. The adjustments in the bicarbonate content are comparatively slow, since they are apparently accomplished by a renal mechanism. This point is illustrated in the experiments on the effects of intravenous administration of sodium salicylate, in which pH and CO_2 tension were markedly altered within a short time, without much change in the total CO_2 content of the serum. Several mechanisms appear possible for the compensatory decrease of bicarbonate. The most efficient and successful compensation (17) would be a reciprocal increase in the concentration of chloride to make up for the lowering of the bicarbonate, thus maintaining the total ionic concentration. Such substitution of chloride for bicarbonate ions, observed repeatedly in man under various conditions, occurred only rarely in the dogs. The removal of bicarbonate, unaccompanied by corresponding increases in other anions,

resulted in lowered total ionic concentrations as indicated by the diminished concentration of sodium and potassium in the blood. In several of the experiments, a diminution of the serum chloride occurred, which was usually accompanied by a marked decrease in the total ionic concentration (sodium + potassium). This decrease was particularly pronounced in animals that had received repeated doses of salicylate, and had been for several days in the state of salicylism. A reduction of the ionic concentration could conceivably occur by way of increased retention of water with consequent dilution of the electrolytes. Actually all animals lost weight and had profuse diuresis. It would appear, therefore, that the animals lost large amounts of sodium, thus offsetting the loss of carbonic acid in the blood by a direct removal of bicarbonate in the urine. Instead of tending to maintain the total electrolyte concentration, such shifts in the electrolyte structure result in cumulative deviations from the normal. The explanation of their mechanism appears difficult. Perhaps the salicylates have an independent action on the kidney which results in impairment of its defense of the osmolarity of the body fluids. The marked diuresis and the albuminuria may be other manifestations of the reno-toxic effect of the salicylates.

The lowered potassium concentration in the serum of the dogs suggests impairment of renal control. The possibility, however, of disturbance of the equilibrium between extra- and intracellular potassium concentrations must be considered. Unpublished data obtained in this laboratory suggest that alkalosis *per se* might exert this effect and also explain lowered concentrations of inorganic phosphorus.

THE EFFECT OF HYPNOTICS ON SALICYLISM

It seems reasonable to assume that the central stimulation caused by the salicylates can be counteracted by hypnotic drugs. In order to test this assumption, experiments were carried out in dogs on the effect of sodium pentobarbital, sodium barbital, paraldehyde, and morphine on the salicylism induced by methyl salicylate. The interaction of sodium barbital and of sodium salicylate was also investigated.

Pentobarbital sodium. In Figure 3 are portrayed the effects of pentobarbital sodium on the

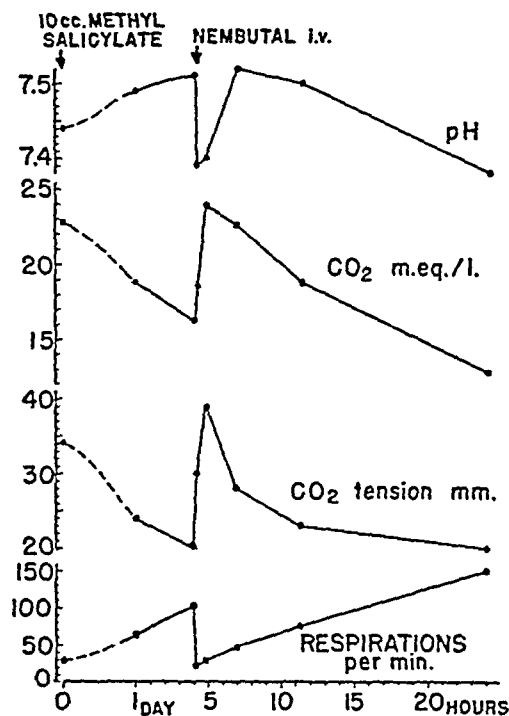


FIG. 3. THE EFFECT OF PENTOBARBITAL SODIUM

Dog, weighing 18 kgm., received 10 ml. of methyl salicylate on the day preceding the intravenous injection of 29 mgm. per kgm. of pentobarbital sodium (nembutal).

pH, the content and tension of CO_2 , and the rate of respiration of a dog which had received in a single dose enough methyl salicylate to produce severe, but not usually fatal, intoxication. On the day following the injection, 2 samples of blood drawn 4 hours apart showed by the progressive alterations of pH and CO_2 that the intoxication was progressing rapidly. Ten minutes after the injection of pentobarbital sodium, the respirations decreased to the normal rate of 20 per minute, and coincidentally the pH fell to a normal value, the serum CO_2 content increased somewhat, and as a result the CO_2 tension rose. A sample of blood taken 50 minutes later showed normal values for all constituents. Two hours after this sample was taken, the respiratory rate had again doubled, the pH had increased to 7.52, the content of CO_2 had dropped to 22.5 m.eq. per liter, and its tension to 28 mm. Hg. Subsequent samples showed decreasing content and tension of the CO_2 . Unlike the animals that received similar doses of salicylates alone, this dog remained in a comatose condition, breathing laboriously, and died 20 hours after the injection of pentobarbital sodium.